Application for United States Letters Patent

To all whom it may concern:

Be it known that we,

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have invented certain new and useful improvements in

DNA ENCODING A $GABA_BR2$ POLYPEPTIDE AND USES THEREOF

of which the following is a full, clear and exact description.

Dkt. 54002-D/JPW/JHB

DNA ENCODING A $GABA_BR2$ POLYPEPTIDE AND USES THEREOF

BACKGROUND OF THE INVENTION

This application is a continuation-in-part of U.S. Serial No. 09/186-004, filed November 4, 1998 which is a continuation-in-part of PCT International Application No. PCT/US98/22033, filed October 16, 1998 which is a continuation-in-part of U.S. Serial No. 09/141,760, filed August 27, 1998, which is a continuation-in-part of U.S. Serial No. 08/953,277, filed October 17, 1997, the contents of which are hereby incorporated by reference into the subject application.

Throughout this application, various references are referred to within parentheses. Disclosures of these publications in their entireties are hereby incorporated by reference into this application to more fully describe the state of the art to which this invention pertains. Full bibliographic citation for these references may be found at the end of this application, preceding the sequence listing and the claims.

Gamma amino butyric acid (GABA) is the major inhibitory neurotransmitter in the nervous system. Three families of receptors for this neurotransmitter, $GABA_A$, $GABA_B$, and $GABA_C$, have been defined pharmacologically and genetically. $GABA_B$ receptors were initially discriminated by their sensitivity to the drug baclofen (Bowery, 1993). This and their dependency on G-proteins for effector coupling distinguishes them from the ion channel-forming $GABA_A$ and $GABA_C$ receptors. Principle molecular targets of $GABA_B$ receptor activation are Ca^{++} and K^+ channels whose gating is directly modulated by the liberation of G-protein that follows the binding of the neurotransmitter to its receptor (Misgeld et al. 1995; Krapivinsky et al., 1995a). In

this sense, $GABA_B$ receptors operate mechanistically as other G-protein coupled receptors (GPCRs), such as dopamine D2, serotonin 5HT1a, neuropeptide Y and opiate receptors, that are also negatively coupled to adenylyl cyclase activity (North, 1989). Stimulation of $GABA_B$ receptors inhibits release of neurotransmitters such as glutamate, GABA, somatostatin, and acetylcholine by modulation of Ca^{++} and K^{+} channels at presynaptic nerve terminals. Inhibition of neurotransmitter release is one of the most prominent physiological actions of the $GABA_B$ receptor and has provided a basis for the discrimination of receptor subtypes (Bowery et al. 1990). $GABA_B$ receptors also mediate a powerful postsynaptic hyperpolarization of neuronal cell bodies via the opening of G-protein-gated inwardly rectifying K^{+} channels (GIRK) (Kofuji et al. 1996).

$GABA_B$ receptors are widely distributed throughout the central nervous system. Receptor autoradiography and binding studies show that receptors are found in relatively high abundance in nearly all areas of the brain including cerebral cortex, hippocampus, cerebellum, basal ganglia, thalamus, and spinal cord (Bowery et al. 1987). In the periphery, GABA and $GABA_B$ receptors are found in pancreatic islets, autonomic ganglia, guinea-pig ileum, lung, oviduct, and urinary bladder (Giotti et al. 1983; Erdo et al. 1984; Santicioli et al. 1986; Sawynok, 1986; Hills et al. 1989; Chapman et al. 1993).

Baclofen, the agonist that originally defined the $GABA_B$ receptor subtype, has been used as an anti-spastic agent for the past 25 years. There is evidence in human that baclofen has a spinal site of action that most likely involves the depression of mono-and polysynaptic reflexes. In laboratory animals, baclofen has antinociceptive properties that are attributed to

the inhibition of release of excitatory neurotransmitters glutamate and substance P from primary sensory afferent terminals (Dirig and Yaksh, 1978; Sawynok, 1987; Malcangio et al., 1991). The presence of $GABA_B$ receptors in intestine, lung and urinary bladder indicates a possible therapeutic role for diseases associated with these peripheral tissues. In spinal patients, baclofen is currently used for treatment of bladder-urethral dissynergia (Leyson et al., 1980). Selective $GABA_B$ receptor agonists may also prove useful for the treatment of incontinence by reducing the feeling of bladder fullness (Taylor and Bates, 1979). Evidence from studies of the upper respiratory systems of cats and guinea-pigs suggests that $GABA_B$ agonists also may be useful as antitussive agents and for the treatment of asthma (Luzzi et al., 1987; Bolser et al., 1993). In addition, $GABA_B$ receptors have been implicated in absence seizure activity in the neocortex and with presynaptic depression of excitatory transmission in the spinal cord.

Studies of $GABA_B$ receptor pharmacology and physiology have been greatly facilitated by the relatively recent arrival of potent and selective $GABA_B$ receptor antagonists that are able to penetrate the blood-brain barrier. The most fruitful avenue for providing glimpses of $GABA_B$ receptor subtypes has come from studies of neurotransmitter release. GABA, acting through $GABA_B$ receptors, can inhibit the release of GABA, glutamate, and somatostatin in rat cerebrocortical synaptosomes depolarized with KCl. Three receptor subtypes have been hypothesized based on the potency of the agonists baclofen and 3-aminopropylphosphinic acid (3-APPA), and on the antagonists phaclofen and CGP35348 (Bonanno, Raiteri, 1992). For example, somatostatin release is inhibited

by baclofen and this effect is antagonized by phaclofen and CGP35348. Glutamate release is similarly affected except that the potency of phaclofen to block inhibition is considerably lower than that for release of somatostatin. A third receptor subtype, the cortical GABA autoreceptor, has been defined based on an insensitivity to CGP35348, although this potency difference is not seen in a cortical slice preparation (Waldmeier et al. 1994). In the spinal cord, the GABA autoreceptor is insensitive to baclofen, but sensitive to 3APPA and block by CGP35348. Interestingly, in this tissue baclofen is active at the $GABA_B$ receptor modulating glutamate release. Differences in the sensitivities of presynaptic receptors controlling release of GABA and glutamate in the spinal cord may importantly contribute to the therapeutic action of baclofen as an antispastic agent (Bonanno, Raiteri, 1993).

Recently a polypeptide was isolated, $GABA_BR1a$, that binds radiolabelled $GABA_B$ receptor antagonists in transfected cells (Kaupmann et al. 1997a). The predicted amino acid sequence displays homology with the metabotropic glutamate receptor gene family which includes eight members and a Ca^{++}-sensing receptor. Included in this homology is a large N-terminal domain that contains two lobes with structural similarity to the amino acid binding sites of bacterial proteins. A second polypeptide, $GABA_BR1b$, presumably a splice variant, differs from $GABA_BR1a$ in that the N-terminal 147 amino acids are replaced by 18 different residues in the predicted mature protein after signal peptide cleavage. Transcripts for both $GABA_BR1s$ are abundant and widely distributed in the rat brain. There appear to be differences in the localization of the splice variants in discrete regions of the brain, suggesting that their expression is differentially regulated

(Bischoff et al. 1997).

The pharmacological profile of the cloned $GABA_BR1$ polypeptide is similar in some respects to that of native receptors isolated from rat cerebral cortex, but there are important differences. For the high affinity antagonists studied, IC_{50}s are nearly identical to those at native receptors. In contrast, IC_{50}s for agonists and some low affinity antagonists display large rightward shifts relative to their displacement curves in native tissue. Additionally, both splice variants of the polypeptide couple poorly to intracellular effectors such as inhibition of adenylyl cyclase and, against expectations, fail completely to stimulate GIRK currents in oocytes (Kaupmann et al. 1997b). The poor binding affinity of agonists and weak or non-existent activation of effectors may not be adequately explained by inappropriate G-protein coupling in the heterologous expression system used.

The isolation by homology cloning of a novel polypeptide, $GABA_BR2$, from a human hippocampus cDNA library, as well the isolation of the rat homolog of the human polypeptide, is now reported. Also reported herein are functional assays involving the co-expression of the $GABA_BR2$ gene with a $GABA_BR1$ gene. These functional assays were not previously observed with the $GABA_BR1$ gene product alone. The pharmacological and signal transduction properties of the two gene products when expressed together match those of native $GABA_B$ receptors in the brain. These functional assays permits high throughput screening for novel compounds having agonist or antagonist activity at the native $GABA_B$ receptor.

SUMMARY OF THE INVENTION

This invention is directed to an isolated nucleic acid encoding a $GABA_BR2$ polypeptide.

This invention is further directed to a purified $GABA_BR2$ protein.

This invention is further directed to a vector comprising the above-identified nucleic acid.

This invention is further directed to a above-identified vector, wherein the vector is a plasmid.

This invention is directed to a method of detecting a nucleic acid encoding a $GABA_BR2$ polypeptide, which comprises contacting the nucleic acid with a probe comprising at least 15 nucleotides, which probe specifically hybridizes with the nucleic acid encoding the $GABA_BR2$ polypeptide, wherein the probe has a unique sequence, which sequence is present within one of the two strands of the nucleic acid encoding the $GABA_BR2$ polypeptide contained in plasmid BO-55, and detecting hybridization of the probe to the nucleic acid.

This invention is further directed to a method of detecting a nucleic acid encoding a $GABA_BR2$ polypeptide, which comprises contacting the nucleic acid with a probe comprising at least 15 nucleotides, which probe specifically hybridizes with the nucleic acid encoding the $GABA_BR2$ polypeptide, wherein the probe has a unique sequence, which sequence is present within (a) the nucleic acid sequence shown in Figures 22A-22D (Seq. ID No. 46) or (b) the reverse complement to the nucleic acid sequence shown in Figures 22A-22D (Seq. ID No. 46), and detecting hybridization of the probe to the nucleic acid.

This invention is further directed to a method of detecting a nucleic acid encoding a $GABA_BR2$ polypeptide, which comprises contacting the nucleic acid with a probe comprising at least 15 nucleotides, which probe specifically hybridizes with the nucleic acid encoding the $GABA_BR2$ polypeptide, wherein the probe has a unique sequence, which sequence is present within one of the two strands of the nucleic acid encoding the $GABA_BR2$ polypeptide contained in plasmid pEXJT3T7-hGABAB2, and detecting hybridization of the probe to the nucleic acid.

This invention is further directed to a method of detecting a nucleic acid encoding a $GABA_BR2$ polypeptide, which comprises contacting the nucleic acid with a probe comprising at least 15 nucleotides, which probe specifically hybridizes with the nucleic acid encoding the $GABA_BR2$ polypeptide, wherein the probe has a unique sequence, which sequence is present within (a) the nucleic acid sequence shown in Figures 3A-3D (Seq. ID No. 3) or (b) the reverse complement to the nucleic acid sequence shown in Figures 3A-3D (Seq. ID No. 3), and detecting hybridization of the probe to the nucleic acid.

This invention is further directed to a method of detecting a nucleic acid encoding a $GABA_BR2$ polypeptide, which comprises contacting the nucleic acid with a probe comprising a nucleic acid of at least 15 nucleotides which is complementary to the antisense sequence of a unique segment of the sequence of the nucleic acid encoding the $GABA_BR2$ polypeptide, and detecting hybridization of the probe to the nucleic acid.

This invention is directed to an isolated antibody capable of binding to a $GABA_BR2$ polypeptide encoded by

the above-identified nucleic acid.

This invention is further directed to an antibody capable of competitively inhibiting the binding of the above-identified antibody to a $GABA_BR2$ polypeptide.

This invention is further directed to a pharmaceutical composition which comprises an amount of the above-identified antibody effective to block binding of a ligand to the $GABA_BR2$ polypeptide and a pharmaceutically acceptable carrier.

This invention is directed to a transgenic, nonhuman mammal expressing DNA encoding a $GABA_BR2$ polypeptide.

This invention is further directed to a transgenic, nonhuman mammal comprising a homologous recombination knockout of the native $GABA_BR2$ polypeptide.

This invention is further directed to a transgenic, nonhuman mammal whose genome comprises antisense DNA complementary to DNA encoding an above-identified $GABA_BR2$ polypeptide so placed as to be transcribed into antisense mRNA which is complementary to mRNA encoding such $GABA_BR2$ polypeptide and which hybridizes to such mRNA encoding such $GABA_BR2$ polypeptide, thereby reducing its translation.

This invention is directed to a method of detecting the presence of a $GABA_BR2$ polypeptide on the surface of a cell which comprises contacting the cell with the above-identified antibody under conditions permitting binding of the antibody to the polypeptide, detecting the presence of the antibody bound to the cell, and thereby detecting the presence of a $GABA_BR2$ polypeptide on the surface of the cell.

This invention is further directed to a method of preparing the purified $GABA_BR2$ polypeptide which comprises:

a. inducing cells to express a $GABA_BR2$ polypeptide;

b. recovering the polypeptide so expressed from the induced cells; and

c. purifying the polypeptide so recovered.

This invention is further directed to a method of preparing the purified $GABA_BR2$ polypeptide which comprises:

a. inserting a nucleic acid encoding the $GABA_BR2$ polypeptide into a suitable vector;

b. introducing the resulting vector in a suitable host cell;

c. placing the resulting cell in suitable condition permitting the production of the $GABA_BR2$ polypeptide;

d. recovering the polypeptide produced by the resulting cell; and

e. isolating or purifying the polypeptide so recovered.

This invention is directed to a $GABA_BR1/R2$ receptor comprising two polypeptides, one of which is a $GABA_BR2$ polypeptide and another of which is a $GABA_BR1$ polypeptide.

This invention is directed to a method of forming a $GABA_BR1/R2$ receptor which comprises inducing cells to express both a $GABA_BR1$ polypeptide and a $GABA_BR2$ polypeptide.

This invention is directed to an antibody capable of binding to a $GABA_BR1/R2$ receptor, wherein the $GABA_BR2$ polypeptide is encoded by the above-identified nucleic acid.

This invention is further directed to an antibody capable of competitively inhibiting the binding of the above-identified antibody to a $GABA_BR1/R2$ receptor.

This invention is directed to a pharmaceutical composition which comprises an amount of the above-identified antibody effective to block binding of a ligand to the $GABA_BR1/R2$ receptor and a pharmaceutically acceptable carrier.

This invention is directed to a transgenic, nonhuman mammal expressing a $GABA_BR1/R2$ receptor, which is not naturally expressed by the mammal.

This invention is further directed to a transgenic, nonhuman mammal comprising a homologous recombination knockout of the native $GABA_BR1/R2$ receptor.

This invention is directed to a method of detecting the presence of a $GABA_BR1/R2$ receptor on the surface of a cell which comprises contacting the cell with the above-identified antibody under conditions permitting binding of the antibody to the receptor, detecting the presence of the antibody bound to the cell, and thereby detecting the presence of a $GABA_BR1/R2$ receptor on the surface of the cell.

This invention is directed to a method of determining the physiological effects of varying levels of activity of $GABA_BR1/R2$ receptors which comprises producing an above-identified transgenic nonhuman mammal whose levels of $GABA_BR1/R2$ receptor activity vary due to the presence of an inducible promoter which regulates $GABA_BR1/R2$ receptor expression.

This invention is directed to a cell which expresses on its surface a mammalian $GABA_BR1/R2$ receptor that is not naturally expressed on the surface of such cell.

This invention is directed to a process for identifying a chemical compound which specifically binds to a $GABA_BR1/R2$ receptor which comprises contacting cells containing nucleic acid encoding and expressing on their cell surface the $GABA_BR1/R2$ receptor, wherein such cells do not normally express the $GABA_BR1/R2$ receptor, with the compound under conditions suitable for binding, and detecting specific binding of the chemical compound to the $GABA_BR1/R2$ receptor.

This invention is directed to a process for identifying a chemical compound which specifically binds to a $GABA_BR1/R2$ receptor which comprises contacting a membrane fraction from a cell extract of cells containing nucleic acid encoding and expressing on their cell surface the $GABA_BR1/R2$ receptor, wherein such cells do not normally express the $GABA_BR1/R2$ receptor, with the compound under conditions suitable for binding, and detecting specific binding of the chemical compound to the $GABA_BR1/R2$ receptor.

This invention is directed to a process involving competitive binding for identifying a chemical compound which specifically binds to a $GABA_BR1/R2$ receptor which comprises separately contacting cells expressing on

their cell surface the $GABA_BR1/R2$ receptor, wherein such cells do not normally express the $GABA_BR1/R2$ receptor, with both the chemical compound and a second chemical compound known to bind to the receptor, and with only the second chemical compound, under conditions suitable for binding of both compounds, and detecting specific binding of the chemical compound to the $GABA_BR1/R2$ receptor, a decrease in the binding of the second chemical compound to the $GABA_BR1/R2$ receptor in the presence of the chemical compound indicating that the chemical compound binds to the $GABA_BR1/R2$ receptor.

This invention is directed to a process involving competitive binding for identifying a chemical compound which specifically binds to a human $GABA_BR1/R2$ receptor which comprises separately contacting a membrane fraction from a cell extract of cells expressing on their cell surface the $GABA_BR1/R2$ receptor, wherein such cells do not normally express the $GABA_BR1/R2$ receptor, with both the chemical compound and a second chemical compound known to bind to the receptor, and with only the second chemical compound, under conditions suitable for binding of both compounds, and detecting specific binding of the chemical compound to the $GABA_BR1/R2$ receptor, a decrease in the binding of the second chemical compound to the $GABA_BR1/R2$ receptor in the presence of the chemical compound indicating that the chemical compound binds to the $GABA_BR1/R2$ receptor.

This invention is directed to a method of screening a plurality of chemical compounds not known to bind to a $GABA_BR1/R2$ receptor to identify a compound which specifically binds to the $GABA_BR1/R2$ receptor, which comprises

(a) contacting cells containing nucleic acid encoding and expressing on their cell surface

the $GABA_BR1/R2$ receptor, wherein such cells do not normally express the $GABA_BR1/R2$ receptor, with a compound known to bind specifically to the $GABA_BR1/R2$ receptor;

(b) contacting the same cells as in step (a) with the plurality of compounds not known to bind specifically to the $GABA_BR1/R2$ receptor, under conditions permitting binding of compounds known to bind the $GABA_BR1/R2$ receptor;

(c) determining whether the binding of the compound known to bind specifically to the $GABA_BR1/R2$ receptor is reduced in the presence of the plurality of the compounds, relative to the binding of the compound in the absence of the plurality of compounds, and if the binding is reduced;

(d) separately determining the extent of binding to the $GABA_BR1/R2$ receptor of each compound included in the plurality of compounds, so as to thereby identify the compound or compounds present in such plurality of compounds which specifically binds to the $GABA_BR1/R2$ receptor.

This invention is directed to a method of screening a plurality of chemical compounds not known to bind to a $GABA_BR1/R2$ receptor to identify a compound which specifically binds to the $GABA_BR1/R2$ receptor, which comprises

(a) contacting a membrane fraction extract from cells containing nucleic acid encoding and expressing on their cell surface the $GABA_BR1/R2$ receptor, wherein such cells do not normally express the $GABA_BR1/R2$ receptor, with

a compound known to bind specifically to the $GABA_BR1/R2$ receptor;

(b) contacting the same membrane fraction as in step (a) with the plurality of compounds not known to bind specifically to the $GABA_BR1/R2$ receptor, under conditions permitting binding of compounds known to bind the $GABA_BR1/R2$ receptor;

(c) determining whether the binding of the compound known to bind specifically to the $GABA_BR1/R2$ receptor is reduced in the presence of the plurality of compounds, relative to the binding of the compound in the absence of the plurality of compounds, and if the binding is reduced;

(d) separately determining the extent of binding to the $GABA_BR1/R2$ receptor of each compound included in the plurality of compounds, so as to thereby identify the compound or compounds present in such plurality of compounds which specifically binds to the $GABA_BR1/R2$ receptor.

This invention is directed to a process for determining whether a chemical compound is a $GABA_BR1/R2$ receptor agonist which comprises contacting cells with the compound under conditions permitting the activation of the $GABA_BR1/R2$ receptor, and detecting an increase in $GABA_BR1/R2$ receptor activity, so as to thereby determine whether the compound is a $GABA_BR1/R2$ receptor agonist.

This invention is directed to a process for determining whether a chemical compound is a $GABA_BR1/R2$ receptor antagonist which comprises contacting cells containing nucleic acid encoding and expressing on their cell

surface the $GABA_BR1/R2$ receptor, wherein such cells do not normally express the $GABA_BR1/R2$ receptor, with the compound in the presence of a known $GABA_BR1/R2$ receptor agonist, under conditions permitting the activation of the $GABA_BR1/R2$ receptor, and detecting a decrease in $GABA_BR1/R2$ receptor activity, so as to thereby determine whether the compound is a $GABA_BR1/R2$ receptor antagonist.

This invention is directed to a process for determining whether a chemical compound activates a $GABA_BR1/R2$ receptor, which comprises contacting cells producing a second messenger response and expressing on their cell surface the $GABA_BR1/R2$ receptor, wherein such cells do not normally express the $GABA_BR1/R2$ receptor, with the chemical compound under conditions suitable for activation of the $GABA_BR1/R2$ receptor, and measuring the second messenger response in the presence and in the absence of the chemical compound, a change in the second messenger response in the presence of the chemical compound indicating that the compound activates the $GABA_BR1/R2$ receptor.

This invention is directed to a process for determining whether a chemical compound inhibits activation of a $GABA_BR1/R2$ receptor, which comprises separately contacting cells producing a second messenger response and expressing on their cell surface the $GABA_BR1/R2$ receptor, wherein such cells do not normally express the $GABA_BR1/R2$ receptor, with both the chemical compound and a second chemical compound known to activate the $GABA_BR1/R2$ receptor, and with only the second chemical compound, under conditions suitable for activation of the $GABA_BR1/R2$ receptor, and measuring the second messenger response in the presence of only the second chemical compound and in the presence of both the second chemical compound and the chemical compound, a

smaller change in the second messenger response in the presence of both the chemical compound and the second chemical compound than in the presence of only the second chemical compound indicating that the chemical compound inhibits activation of the $GABA_BR1/R2$ receptor.

This invention is directed to a method of screening a plurality of chemical compounds not known to activate a $GABA_BR1/R2$ receptor to identify a compound which activates the $GABA_BR1/R2$ receptor which comprises:

(a) contacting cells containing nucleic acid encoding and expressing on their cell surface the $GABA_BR1/R2$ receptor, wherein such cells do not normally express the $GABA_BR1/R2$ receptor, with the plurality of compounds not known to activate the $GABA_BR1/R2$ receptor, under conditions permitting activation of the $GABA_BR1/R2$ receptor;

(b) determining whether the activity of the $GABA_BR1/R2$ receptor is increased in the presence of the compounds, and if it is increased;

(c) separately determining whether the activation of the $GABA_BR1/R2$ receptor is increased by each compound included in the plurality of compounds, so as to thereby identify the compound or compounds present in such plurality of compounds which activates the $GABA_BR1/R2$ receptor.

This invention is directed to a method of screening a plurality of chemical compounds not known to inhibit the activation of a $GABA_BR1/R2$ receptor to identify a compound which inhibits the activation of the $GABA_BR1/R2$

receptor, which comprises:

(a) contacting cells containing nucleic acid encoding and expressing on their cell surface the $GABA_BR1/R2$ receptor, wherein such cells do not normally express the $GABA_BR1/R2$ receptor, with the plurality of compounds in the presence of a known $GABA_BR1/R2$ receptor agonist, under conditions permitting activation of the $GABA_BR1/R2$ receptor;

(b) determining whether the activation of the $GABA_BR1/R2$ receptor is reduced in the presence of the plurality of compounds, relative to the activation of the $GABA_BR1/R2$ receptor in the absence of the plurality of compounds, and if it is reduced;

(c) separately determining the inhibition of activation of the $GABA_BR1/R2$ receptor for each compound included in the plurality of compounds, so as to thereby identify the compound or compounds present in such a plurality of compounds which inhibits the activation of the $GABA_BR1/R2$ receptor.

This invention is directed to a process for determining whether a chemical compound is a $GABA_BR1/R2$ receptor agonist, which comprises preparing a membrane fraction from cells which comprise nucleic acid encoding and expressing on their cell surface the $GABA_BR1/R2$ receptor, wherein such cells do not normally express the $GABA_BR1/R2$ receptor, separately contacting the membrane fraction with both the chemical compound and $GTP\gamma S$, and with only $GTP\gamma S$, under conditions permitting the activation of the $GABA_BR1/R2$ receptor, and detecting $GTP\gamma S$ binding to the membrane fraction, an increase in

GTPγS binding in the presence of the compound indicating that the chemical compound activates the $GABA_BR1/R2$ receptor.

This invention is directed to aprocess for determining whether a chemical compound is a $GABA_BR1/R2$ receptor antagonist, which comprises preparing a membrane fraction from cells which comprise nucleic acid encoding and expressing on their cell surface the $GABA_BR1/R2$ receptor, wherein such cells do not normally express the $GABA_BR1/R2$ receptor, separately contacting the membrane fraction with the chemical compound, GTPγS and a second chemical compound known to activate the $GABA_BR1/R2$ receptor, with GTPγS and only the second compound, and with GTPγS alone, under conditions permitting the activation of the $GABA_BR1/R2$ receptor, detecting GTPγS binding to each membrane fraction, and comparing the increase in GTPγS binding in the presence of the compound and the second compound relative to the binding of GTPγS alone, to the increase in GTPγS binding in the presence of the second chemical compound known to activate the $GABA_BR1/R2$ receptor relative to the binding of GTPγS alone, a smaller increase in GTPγS binding in the presence of the compound and the second compound indicating that the compound is a $GABA_BR1/R2$ receptor antagonist.

This invention is directed to a method of treating spasticity in a subject which comprises administering to the subject an amount of a compound which is an agonist of a $GABA_BR1/R2$ receptor effective to treat spasticity in the subject.

This invention is directed to a method of treating asthma in a subject which comprises administering to the subject an amount of a compound which is a $GABA_BR1/R2$ receptor agonist effective to treat asthma in

the subject.

This invention is directed to a method of treating incontinence in a subject which comprises administering to the subject an amount of a compound which is a $GABA_BR1/R2$ receptor agonist effective to treat incontinence in the subject.

This invention is directed to a method of decreasing nociception in a subject which comprises administering to the subject an amount of a compound which is a $GABA_BR1/R2$ receptor agonist effective to decrease nociception in the subject.

This invention is directed to a use of a $GABA_BR2$ agonist as an antitussive agent which comprises administering to the subject an amount of a compound which is a $GABA_BR1/R2$ receptor agonist effective as an antitussive agent in the subject.

This invention is directed to a method of treating drug addiction in a subject which comprises administering to the subject an amount of a compound which is a $GABA_BR1/R2$ receptor agonist effective to treat drug addiction in the subject.

This invention is directed to a method of treating Alzheimer's disease in a subject which comprises administering to the subject an amount of a compound which is a $GABA_BR1/R2$ receptor antagonist effective to treat Alzheimer's disease in the subject.

This invention is directed to a peptide selected from the group consisting of:

a) P L Y S I L S A L T I L G M I M A S A F L F F N I K N; (SEQ ID NO:46)

b) L I I L G G M L S Y A S I F L F G L D G S F V S

(SEQ ID NO:49)

E K T;^

c) C T V R T W I L T V G Y T T A F G A M F A K T W R;^ (SEQ ID NO:50)

d) Q K L L V I V G G M L L I D L C I L I C W Q;^ (SEQ ID NO:51)

e) M T I W L G I V Y A Y K G L L M L F G C F L A W;^ (SEQ ID NO:52)

f) A L N D S K Y I G M S V Y N V G I M C I I G A A V;^ (SEQ ID NO:53) and

g) C I V A L V I I F C S T I T L C L V F V P K L I T L R T N;^ (SEQ ID NO:54)

This invention is directed to a compound that prevents the formation of a $GABA_B$R1/R2 receptor complex.

Finally, this invention provides a process for making a composition of matter which specifically binds to a $GABA_B$R1/R2 receptor which comprises identifying a chemical compound using any of the processes described herein for identifying a compound which binds to and/or activates or inhibits activation of a $GABA_B$R1/R2 receptor and then synthesizing the chemical compund or a novel structural and functional analog or homolog thereof. This invention furhter provides a process for preparing a pharmaceutical composition which comprises admixing a pharmaceutically acceptable carrier and a pharmaceutically acceptable amount of a chemical compound identified by any of the processes described herein for identifying a compound which binds to and/or activates or inhibits activation of a $GABA_B$R1/R2 receptor or a novel structural and functional analog or homolog thereof.

BRIEF DESCRIPTION OF THE FIGURES

Figures 1A-1E Nucleotide coding sequence of the human $GABA_BR2$ polypeptide (Seq. ID No. 1), with partial 5' and 3' untranslated sequences. Two possible start (ATG) codons are underlined as well as the stop codon (TAA).

Figures 2A-2D Deduced amino acid sequence of the human $GABA_BR2$ polypeptide (Seq. ID No. 2) encoded by the nucleotide sequence shown in Figures 1A-1E.

Figures 3A-3D Nucleotide coding sequence of the rat $GABA_BR2$ polypeptide (Seq. ID No. 3). Start (ATG) and stop (TAG) codons are underlined.

Figures 4A-4D Deduced amino acid sequence of the rat $GABA_BR2$ polypeptide (Seq. ID No. 4) encoded by the nucleotide sequence shown in Figures 3A-3D.

Figures 5A-5D Amino acid sequence of the human $GABA_BR2$ polypeptide (Seq. ID No. 2) with brackets above the sequence showing the boundaries of seven (7) putative transmembrane domains, numbered consecutively from I to VII.

Figures 6A-6B. Measurement of EC_{50} for GABA in a cumulative concentration response assay in oocytes expressing $GABA_BR1b/GABA_BR2$ + GIRKs. Figure 6A: Electrophysiological trace from a voltage clamped oocyte showing increasing inward currents evoked successively by concentrations of GABA ranging from 0.03 to 30 μM. Numbers over bars indicate concentration of GABA in μM. hK is 49 mM external K^+. Figure 6B: Averaged responses from 3-6 oocytes plotted *vs.* concentration of GABA results in an EC_{50} value of 1.76 μM. For each oocyte, currents were normalized to the maximum response at 30 μM.

Figure 7. Concentration response relationship for baclofen in oocytes expressing $GABA_BR1b/GABA_BR2$ + GIRKs. Methods are as described for Figure 6.

Figure 8. Current voltage relationship for the current activated by GABA in oocytes expressing $GABA_BR1b/GABA_BR2$ + GIRKs. Voltage ramps (50 mV/s) from -140 to +40 mV were applied in the presence of GABA (in hK) and again in the presence of GABA + 100 μM Ba^{++} to block inward rectifier current. The resulting traces were subtracted (GABA alone - GABA + Ba^{++}) to yield the Ba^{++}-sensitive portion of the GABA-stimulated current. As expected for GIRK current, the current displays steep inward rectification and reverses near the predicted equilibrium potential for K+ (-23 mV in hK).

Figures 9A-9B. Electrophysiological responses under voltage clamp conditions to GABA in an HEK-293 cell transiently transfected with $GABA_BR1b/GABA_BR2$ + GIRKs. A) The continuous trace (in presence of 25 mM K^+) shows a small constitutive inward rectifier current that is blocked by Ba^{++} (100 μM), and a much larger inward current induced by application of GABA that is also blocked by Ba^{++}. A second GABA-evoked current is abolished by the selective antagonist CGP55845. After a 1 minute wash period GABA-responsivity returns. B) Concentration response relation for GABA in 5 HEK-293 cells expressing $GABA_BR1b/GABA_BR2$ + GIRKs. (See Figure 6B for details.)

Figure 10. Alignment of amino acid s predicted for rat $GABA_BR2$ (SEQ ID NO:4) and rat $GABA_BR1$ (SEQ ID NO:55). Horizontal bars indicate TM regions.

Figures 11A-11D. Photomicrographs showing the regional distribution of the $GABA_BR1$ (A,C) and $GABA_BR2$ (B,D) mRNAs in representative coronal rat brain sections.

Hypothalamus and caudate-putamen are identified with arrow heads and arrows, respectively (A,B). Arrows identify Purkinje cell layer in cerebellum (C,D).

Figures 12A-12B. High magnification micrographs of Purkinje cell layer from alternate serial sections showing co-localization of $GABA_BR2$ transcripts using digoxigenin-labeled probes (A) and $GABA_BR1$ transcripts using $[^{35}S]$dATP-labeled probes (B) in the same cells (asterisks). Scale bar = 30 μM.

Figures 13A-13B. Figure 13A: Response to GABA (100 μM) from oocyte expressing $GABA_BR1$, $GABA_BR2$, and GIRKs (lower trace). Similar oocyte pretreated 6 h earlier with pertussis toxin (2 ng injected; upper trace). Figure 13B: Summary of mean response amplitudes from oocytes expressing various combinations of $GABA_BR1$ and $GABA_BR2$ plus GIRKs. Responses are to 100 μM GABA (solid bars) or 100 μM baclofen (open bar). Number of observations are in parenthesis.

Figures 14A-14B. Figure 14A: Response to GABA or baclofen (100 μM in 25 mM K^+) in HEK293 cells expressing GIRKs along with $GABA_BR1b$, $GABA_BR2$, or both. Figure 14B: Summary of mean response amplitudes from HEK293 cells co-transfected with various combinations and ratios of cDNA. To prepare different ratios of $GABA_BR1b$:$GABA_BR2$ the most abundant cDNA was held constant at 0.6 μg/dish and the other cDNA was reduced by a factor of 10 or 100. Responses are to 100 μM GABA. Number of observations are shown in parenthesis.

Figures 15A-15B. Figure 15A: Agonist concentration-effect curves for 3-APMPA in oocytes (open triangle), GABA in oocytes (open circle) and HEK293 cells (solid circle), and baclofen in oocytes (open square). Figure 15B: Right-ward shifts in the GABA concentration-

response curve (solid circle) caused by CGP55845 at 50 nM (open triangle) and CGP54626 at 5 μM (open circle). Each point is the average response from 4-6 oocytes.

Figure 16. Microphysiometric response to baclofen (100 μM) from CHO cells expressing combinations of $GABA_BR1$ and $GABA_BR2$ (n = 4).

Figures 17A-17D. Co-localization of $GABA_BR1$ and $GABA_BR2$ in HEK293 cells by dual wavelength scanning confocal microscopy. Figure 17A: Green channel showing $GABA_BR1^{RGS6xH}$ (labeled with FITC) in cell expressing both $GABA_BR1^{RGS6xH}$ and $GABA_BR2^{HA}$. Figure 17B: Red channel showing $GABA_BR2^{HA}$ (labeled with TRITC) localization in the same cell. Figure 17C: Dual channel image of the same cell reveals a predominant yellow hue caused by the co-localization of fluorescent tags for $GABA_BR1^{RGS6xH}$ and $GABA_BR2^{HA}$. Figure 17D: Dual wavelength image of cell expressing $GABA_BR2^{HA}$ (red) and NPY $Y5^{Flag}$ (green). Note the low degree of spatial overlap of the two polypeptides.

Figures 18A-18C. Identification of $GABA_BR1$ and $GABA_BR2$ in cell lysates and immunoprecipitates. Figure 18A: Detection of $GABA_BR1^{RGS6xH}$ in whole cell extracts from cells expressing either or both polypeptides. Proteins labeled with anti-His or anti-HA, migrate as monomeric and dimeric forms. Figure 18B: Detection of $GABA_BR2^{HA}$ in whole cell extracts from cells expressing either or both. Labels over lanes denote which polypeptides were transfected. Proteins labeled with anti-His or anti-HA, migrate as monomeric and dimeric forms. Figure 18C: Co-immunoprecipitation of $GABA_BR1^{RGS6xH}$ and $GABA_BR2^{HA}$. Variously transfected cells were immunoprecipitated (IP) with anti-HA or anti-His antibodies, subjected to SDS-PAGE, blotted, and probed for the presence of the HA epitope. Note that in anti-His immunoprecipitated

material, HA immunoreactivity appears only in the lane from cells expressing both proteins.

Figure 19A-19I

~~Figure 19.~~ Rostro-caudal distribution of the $GABA_BR2$ mRNA in coronal rat brain sections (A-F) and spinal cord (G). Brightfield photomicrographs of the dorsal root (H) and trigeminal (I) ganglia showing silver grains over the cells indicating the presence of $GABA_BR2$ mRNA.

Figure 20A-20C

~~Figure 20.~~ (A) Detection of Na+/K+ ATPase by anti-alpha 1 subunit antibodies in membrane fractions enriched in (P1+) or depleted of (P2) plasma membranes (50 :g protein/lane). (B) Co-immunoprecipitation of $GABA_BR1^{RGS6xH}$ and $GABA_BR2^{HA}$ from solubilized P1+ membrane fractions. Note that in anti-His immunoprecipitated material, HA immunoreactivity appears only in the lane from cells expressing both proteins. (C) Western blot showing enrichment of $GABA_BR2^{HA}$ in P1+ membrane fraction as compared to the P2 fraction.

Figure 21A-21F

~~Figure 21~~. Photomicrographs showing the regional distribution of $GABA_BR2$ (A,C) and $GABA_BR1b$ (B,D) mRNAs in pairs of adjacent coronal rat brain sections. Arrow heads identify Purkinje cell layer in cerebellum (A,B). High magnification views of hippocampal CA3 region showing both transcripts in cells from alternate sections (C,D). Arrows mark individual cells. Hybridization of $GABA_BR2$ (E) and $GABA_BR1b$ (F) transcripts in large cells of mesencephalic trigeminal nucleus.

Figure 22A-22D Nucleotide coding sequence of the human $GABA_BR2$ polypeptide (Seq. ID No. 46). Start (ATG) and stop (TAA) codons are underlined.

Figure 23A-23D Deduced amino acid sequence of the human $GABA_BR2$ polypeptide (Seq. ID No. 47) encoded by the

nucleotide sequence shown in Figures 22A-22D.

add E^2

DETAILED DESCRIPTION OF THE INVENTION

In this application, the following standard abbreviations are used to indicate specific nucleotide bases:

C = cytosine	A = adenine
T = thymine	G = guanine

In this application, the term 7-TM spanning protein or a 7-TM protein indicates a protein presumed to have seven transmembrane regions which cross the cellular membrane band on its amino acid sequence.

This invention is directed to an isolated nucleic acid encoding a $GABA_BR2$ polypeptide.

In one embodiment, the nucleic acid is DNA. In another embodiment, the DNA is cDNA. In another embodiment, the DNA is genomic DNA. In another embodiment, the nucleic acid is RNA. In another embodiment, the nucleic acid encodes a mammalian $GABA_BR2$ polypeptide. In another embodiment, the nucleic acid encodes a rat $GABA_BR2$ polypeptide. In another embodiment, the nucleic acid encodes a human $GABA_BR2$ polypeptide.

In another embodiment, the nucleic acid encodes a polypeptide characterized by an amino acid sequence in the transmembrane regions which has an identity of 90% or higher to the amino acid sequence in the transmembrane regions of the human $GABA_BR2$ polypeptide shown in Figures 5A-5D.

In another embodiment, the nucleic acid encodes a mammalian $GABA_BR2$ polypeptide which has substantially the same amino acid sequence as does the $GABA_BR2$ polypeptide encoded by the plasmid BO-55 (ATCC

Accession No. 209104). In another embodiment, the nucleic acid encodes a rat $GABA_BR2$ polypeptide which has an amino acid sequence encoded by the plasmid BO-55 (ATCC Accession No. 209104).

In another embodiment, the nucleic acid encodes a rat $GABA_BR2$ polypeptide having substantially the same amino acid sequence as the amino acid sequence shown in Figures 4A-4D (Seq. ID No. 4). In another embodiment, the nucleic acid encodes a rat $GABA_BR2$ polypeptide having the amino acid sequence shown in Figures 4A-4D (Seq. ID No. 4).

In another embodiment, the nucleic acid encodes a mammalian $GABA_BR2$ polypeptide which has substantially the same amino acid sequence as does the $GABA_BR2$ polypeptide encoded by the plasmid pEXJT3T7-hGABAB2 (ATCC Accession No. 203515). In another embodiment, the nucleic acid encodes a human $GABA_BR2$ polypeptide which has an amino acid sequence encoded by the plasmid pEXJT3T7-hGABAB2 (ATCC Accession No. 203515).

In another embodiment, the human $GABA_BR2$ polypeptide has a sequence, which sequence comprises substantially the same amino acid sequence as the sequence shown in Figures 23A-23D (Seq. ID No. 47).

In another embodiment, the human $GABA_BR2$ polypeptide has a sequence, which sequence comprises the sequence shown in Figures 23A-23D (Seq. ID No. 47).

This application further supports an isolated nucleic acid encoding a $GABA_BR2$ polypeptide, the amino acid sequence of which is encoded by the nucleotide sequence set forth in either the Figures 22A-22D and 3A-3D.

Further, the human $GABA_BR2$ polypeptide described herein

exhibits 38% amino acid identity with the $GABA_BR1a$ polypeptide, while the rat $GABA_BR2$ polypeptide described herein exhibits 98% identity with the human $GABA_BR2$ polypeptide.

The ATG encoding the methionine at position 16 is surrounded by flanking sequences which correspond to the well-known Kozak consensus sequence for translation initiation (Kozak, 1989 and Kozak, 1991), thus the sequence from amino acid 16 through amino acid 898 is believed to be the most likely polypeptide expressed by the nucleic acid. Neither the ATG encoding methionine 1 nor the ATG encoding methionine 19 has the Kozak flanking sequences; however, it is to be understood that the present invention provides a $GABA_BR2$ polypeptide having any one of the three possible starting methionines.

This invention provides a splice variant of the polypeptides disclosed herein. This invention further provides for alternate translation initiation sites and alternately spliced or edited variants of nucleic acids encoding rat and human polypeptides of this invention.

Methods for production and manipulation of nucleic acid molecules are well known in the art.

This invention also encompasses DNAs and cDNAs which encode amino acid sequences which differ from those of the polypeptides of this invention, but which should not produce phenotypic changes. Alternatively, this invention also encompasses DNAs, cDNAs, and RNAs which hybridize to the DNA, cDNA, and RNA of the subject invention. Hybridization methods are well known to those of skill in the art.

The nucleic acids of the subject invention also include

nucleic acid molecules coding for polypeptide analogs, fragments or derivatives of antigenic polypeptides which differ from naturally-occurring forms in terms of the identity or location of one or more amino acid residues (deletion analogs containing less than all of the residues specified for the protein, substitution analogs wherein one or more residues specified are replaced by other residues and addition analogs where in one or more amino acid residues is added to a terminal or medial portion of the polypeptides) and which share some or all properties of naturally-occurring forms. These molecules include: the incorporation of codons "preferred" for expression by selected non-mammalian hosts; the provision of sites for cleavage by restriction endonuclease enzymes; and the provision of additional initial, terminal or intermediate DNA sequences that facilitate construction of readily expressed vectors.

The modified polypeptides of this invention may be transfected into cells either transiently or stably using methods well-known in the art, examples of which are disclosed herein.

This invention also provides for binding assays using the modified polypeptides, in which the polypeptide is expressed either transiently or in stable cell lines. This invention further provides for a compound identified using a modified polypeptide in a binding assay such as the binding assays described herein.

The nucleic acids described and claimed herein are useful for the information which they provide concerning the amino acid sequence of the polypeptide and as products for the large scale synthesis of the polypeptide by a variety of recombinant techniques. The nucleic acid molecule is useful for generating new

cloning and expression vectors, transformed and transfected prokaryotic and eukaryotic host cells, and new and useful methods for cultured growth of such host cells capable of expression of the polypeptide and related products.

Vectors which comprise the isolated nucleic acid molecule described hereinabove also are provided. Suitable vectors comprise, but are not limited to, a plasmid or a virus. These vectors may be transformed into a suitable host cell to form a host cell expression system for the production of a $GABA_BR2$ polypeptide. Suitable host cells include, for example, neuronal cells such as the glial cell line C6, a Xenopus cell such as an oocyte or melanophore cell, as well as numerous mammalian cells and non-neuronal cells.

This invention further provides for any vector or plasmid which comprises modified untranslated sequences, which are beneficial for expression in desired host cells or for use in binding or functional assays. For example, a vector or plasmid with untranslated sequences of varying lengths may express differing amounts of the polypeptide depending upon the host cell used. In an embodiment, the vector or plasmid comprises the coding sequence of the polypeptide and the regulatory elements necessary for expression in the host cell.

As used herein, the phrase "specifically hybridizing" means the ability of a nucleic acid molecule to recognize a nucleic acid sequence complementary to its own and to form double-helical segments through hydrogen bonding between complementary base pairs. The term "complementary" is used in its usual sense in the art, i.e., G and C are complementary and A is

complementary to T (or U in RNA), such that two strands of nucleic acid are "complementary" only if every base matches the opposing base exactly.

This invention is directed to a purified $GABA_BR2$ protein.

This invention is directed to a vector comprising a above-identified nucleic acid.

In one embodiment, the vector is adapted for expression in a bacterial cell which comprises the regulatory elements necessary for expression of the nucleic acid in the bacterial cell operatively linked to the nucleic acid encoding a $GABA_BR2$ polypeptide so as to permit expression thereof.

In another embodiment, the vector is adapted for expression in an amphibian cell which comprises the regulatory elements necessary for expression of the nucleic acid in the amphibian cell operatively linked to the nucleic acid encoding a $GABA_BR2$ polypeptide so as to permit expression thereof.

In another embodiment, the vector is adapted for expression in a yeast cell which comprises the regulatory elements necessary for expression of the nucleic acid in the yeast cell operatively linked to the nucleic acid encoding a $GABA_BR2$ polypeptide so as to permit expression thereof.

In another embodiment, the vector is adapted for expression in an insect cell which comprises the regulatory elements necessary for expression of the nucleic acid in the insect cell operatively linked to the nucleic acid encoding the $GABA_BR2$ polypeptide so as to permit expression thereof.

In one embodiment, the vector is a baculovirus.

In another embodiment, the vector is adapted for expression in a mammalian cell which comprises the regulatory elements necessary for expression of the nucleic acid in the mammalian cell operatively linked to the nucleic acid encoding a $GABA_BR2$ polypeptide so as to permit expression thereof.

In one embodiment, the vector is a plasmid.

In a further embodiment, the plasmid is designated BO-55 (ATCC Accession No. 209104).

In a further embodiment, the plasmid is designated pEXJT3T7-hGABAB2 (ATCC Accession No. 203515).

This invention provides a plasmid designated pEXJT3T7-hGABAB2 (ATCC Accession No. 203515) which comprises the regulatory elements necessary for expression of DNA in a mammalian cell operatively linked to DNA encoding the human polypeptide so as to permit expression thereof.

This plasmid (pEXJT3T7-hGABAB2) was deposited on December 30, 1998, with the American Type Culture Collection (ATCC), 10801 University Blvd., Manassas, Virginia 20110-2209, U.S.A. under the provisions of the Budapest Treaty for the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure and was accorded ATCC Accession No. 203515.

This invention provides a plasmid designated BO-55 (ATCC Accession No. 209104) which comprises the regulatory elements necessary for expression of DNA in a mammalian cell operatively linked to DNA encoding the rat polypeptide so as to permit expression thereof.

This plasmid (BO-55) was deposited on June 10, 1997, with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A. under the provisions of the Budapest Treaty for the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure and was accorded ATCC Accession No. 209104.

Nucleic acid probe technology is well known to those skilled in the art who will readily appreciate that such probes may vary greatly in length and may be labeled with a detectable label, such as a radioisotope or fluorescent dye, to facilitate detection of the probe. DNA probe molecules may be produced by insertion of a DNA molecule which encodes the polypeptides of this invention into suitable vectors, such as plasmids or bacteriophages, followed by transforming into suitable bacterial host cells, replication in the transformed bacterial host cells and harvesting of the DNA probes, using methods well known in the art. Alternatively, probes may be generated chemically from DNA synthesizers.

RNA probes may be generated by inserting the DNA molecule which encodes the polypeptides of this invention downstream of a bacteriophage promoter such as T3, T7 or SP6. Large amounts of RNA probe may be produced by incubating the labeled nucleotides with the linearized fragment where it contains an upstream promoter in the presence of the appropriate RNA polymerase.

This invention is directed to a method of detecting a nucleic acid encoding a $GABA_BR2$ polypeptide, which comprises contacting the nucleic acid with a probe comprising at least 15 nucleotides, which probe specifically hybridizes with the nucleic acid encoding

the $GABA_BR2$ polypeptide, wherein the probe has a unique sequence, which sequence is present within one of the two strands of the nucleic acid encoding the $GABA_BR2$ polypeptide contained in plasmid BO-55, and detecting hybridization of the probe to the nucleic acid.

This invention is directed to a method of detecting a nucleic acid encoding a $GABA_BR2$ polypeptide, which comprises contacting the nucleic acid with a probe comprising at least 15 nucleotides, which probe specifically hybridizes with the nucleic acid encoding the $GABA_BR2$ polypeptide, wherein the probe has a unique sequence, which sequence is present within (a) the nucleic acid sequence shown in Figures 22A-22D (Seq. ID No. 46) or (b) the reverse complement to the nucleic acid sequence shown in Figures 22A-22D (Seq. ID No. 46), and detecting hybridization of the probe to the nucleic acid.

This invention is directed to a method of detecting a nucleic acid encoding a $GABA_BR2$ polypeptide, which comprises contacting the nucleic acid with a probe comprising at least 15 nucleotides, which probe specifically hybridizes with the nucleic acid encoding the $GABA_BR2$ polypeptide, wherein the probe has a unique sequence, which sequence is present within one of the two strands of the nucleic acid encoding the $GABA_BR2$ polypeptide contained in plasmid pEXJT3T7-hGABAB2 and detecting hybridization of the probe to the nucleic acid.

This invention is directed to a method of detecting a nucleic acid encoding a $GABA_BR2$ polypeptide, which comprises contacting the nucleic acid with a probe comprising at least 15 nucleotides, which probe specifically hybridizes with the nucleic acid encoding the $GABA_BR2$ polypeptide, wherein the probe has a unique

sequence, which sequence is present within (a) the nucleic acid sequence shown in Figures 3A-3D (Seq. ID No. 3) or (b) the reverse complement to the nucleic acid sequence shown in Figures 3A-3D (Seq. ID No. 3), and detecting hybridization of the probe to the nucleic acid.

In one embodiment, the nucleic acid is DNA.

In another embodiment, the nucleic acid is RNA.

In one embodiment, the probe comprises at least 15 nucleotides complementary to a unique segment of the sequence of the nucleic acid molecule encoding the $GABA_BR2$ polypeptide.

This invention is directed to a method of detecting a nucleic acid encoding a $GABA_BR2$ polypeptide, which comprises contacting the nucleic acid with a probe comprising a nucleic acid of at least 15 nucleotides which is complementary to the antisense sequence of a unique segment of the sequence of the nucleic acid encoding the $GABA_BR2$ polypeptide, and detecting hybridization of the probe to the nucleic acid.

This invention is directed to a method of inhibiting translation of mRNA encoding a $GABA_BR2$ polypeptide which comprises contacting such mRNA with an antisense oligonucleotide having a sequence capable of specifically hybridizing to the above-identified mRNA, so as to prevent translation of the mRNA.

This invention is directed to a method of inhibiting translation of mRNA encoding a $GABA_BR2$ polypeptide which comprises contacting such mRNA with an antisense oligonucleotide having a sequence capable of specifically hybridizing to the above-identified

genomic DNA.

In one embodiment, the oligonucleotide comprises chemically modified nucleotides or nucleotide analogues.

In another embodiment, the isolated antibody is capable of binding to a $GABA_BR2$ polypeptide encoded by an above-identified nucleic acid.

In another embodiment, the $GABA_BR2$ polypeptide is a human $GABA_BR2$ polypeptide.

This invention is directed to an antibody capable of competitively inhibiting the binding of an above-identified antibody to a $GABA_BR2$ polypeptide.

In one embodiment, the antibody is a monoclonal antibody.

In one embodiment, the monoclonal antibody is directed to an epitope of a $GABA_BR2$ polypeptide present on the surface of a $GABA_BR2$ polypeptide expressing cell.

In another embodiment, the oligonucleotide is coupled to a substance which inactivates mRNA.

In another embodiment, the substance which inactivates mRNA is a ribozyme.

This invention is directed to a pharmaceutical composition which comprises an amount of an above-identified antibody effective to block binding of a ligand to the $GABA_BR2$ polypeptide and a pharmaceutically acceptable carrier.

As used herein, "pharmaceutically acceptable carriers"

means any of the standard pharmaceutically acceptable carriers. Examples include, but are not limited to, phosphate buffered saline, physiological saline, water and emulsions, such as oil/water emulsions.

Animal model systems which elucidate the physiological and behavioral roles of the polypeptides of this invention are produced by creating transgenic animals in which the activity of the polypeptide is either increased or decreased, or the amino acid sequence of the expressed polypeptide is altered, by a variety of techniques. Examples of these techniques include, but are not limited to: 1) Insertion of normal or mutant versions of DNA encoding the polypeptide, by microinjection, electroporation, retroviral transfection or other means well known to those skilled in the art, into appropriate fertilized embryos in order to produce a transgenic animal or 2) Homologous recombination of mutant or normal, human or animal versions of these genes with the native gene locus in transgenic animals to alter the regulation of expression or the structure of these polypeptide sequences. The technique of homologous recombination is well known in the art. It replaces the native gene with the inserted gene and so is useful for producing an animal that cannot express native polypeptides but does express, for example, an inserted mutant polypeptide, which has replaced the native polypeptide in the animal's genome by recombination, resulting in underexpression of the transporter. Microinjection adds genes to the genome, but does not remove them, and so is useful for producing an animal which expresses its own and added polypeptides, resulting in overexpression of the polypeptides.

One means available for producing a transgenic animal, with a mouse as an example, is as follows: Female mice

are mated, and the resulting fertilized eggs are dissected out of their oviducts. The eggs are stored in an appropriate medium such as M2 medium. DNA or cDNA encoding a polypeptide of this invention is purified from a vector by methods well known in the art. Inducible promoters may be fused with the coding region of the DNA to provide an experimental means to regulate expression of the trans-gene. Alternatively, or in addition, tissue specific regulatory elements may be fused with the coding region to permit tissue-specific expression of the trans-gene. The DNA, in an appropriately buffered solution, is put into a microinjection needle (which may be made from capillary tubing using a pipet puller) and the egg to be injected is put in a depression slide. The needle is inserted into the pronucleus of the egg, and the DNA solution is injected. The injected egg is then transferred into the oviduct of a pseudopregnant mouse (a mouse stimulated by the appropriate hormones to maintain pregnancy but which is not actually pregnant), where it proceeds to the uterus, implants, and develops to term. As noted above, microinjection is not the only method for inserting DNA into the egg cell, and is used here only for exemplary purposes.

This invention is directed to a transgenic, nonhuman mammal expressing DNA encoding a $GABA_BR2$ polypeptide.

This invention is directed to a transgenic, nonhuman mammal comprising a homologous recombination knockout of the native $GABA_BR2$ polypeptide.

This invention is further directed to a transgenic, nonhuman mammal whose genome comprises antisense DNA complementary to DNA encoding a $GABA_BR2$ polypeptide so placed as to be transcribed into antisense mRNA which is complementary to mRNA encoding such $GABA_BR2$

polypeptide and which hybridizes to such mRNA encoding such $GABA_BR2$ polypeptide, thereby reducing its translation.

This invention is directed to an above-identified transgenic, nonhuman mammal, wherein the DNA encoding the $GABA_BR2$ polypeptide additionally comprises an inducible promoter.

This invention is directed to an above-identified transgenic, nonhuman mammal, wherein the DNA encoding the $GABA_BR2$ polypeptide additionally comprises tissue specific regulatory elements.

This invention is directed to an above-identified transgenic, nonhuman mammal, wherein the transgenic, nonhuman mammal is a mouse.

This invention is directed to method of detecting the presence of a $GABA_BR2$ polypeptide on the surface of a cell which comprises contacting the cell with an above-identified antibody under conditions permitting binding of the antibody to the polypeptide, detecting the presence of the antibody bound to the cell, and thereby detecting the presence of a $GABA_BR2$ polypeptide on the surface of the cell.

This invention is directed to a method of preparing a purified $GABA_BR2$ polypeptide which comprises:

a. inducing cells to express a $GABA_BR2$ polypeptide;

b. recovering the polypeptide so expressed from the induced cells; and

c. purifying the polypeptide so recovered.

This invention is directed to a method of preparing the purified $GABA_BR2$ polypeptide which comprises:

a. inserting a nucleic acid encoding the $GABA_BR2$ polypeptide into a suitable vector;

b. introducing the resulting vector in a suitable host cell;

c. placing the resulting cell in suitable condition permitting the production of the $GABA_BR2$ polypeptide;

d. recovering the polypeptide produced by the resulting cell; and

e. isolating or purifying the polypeptide so recovered.

This invention is directed to a $GABA_BR1/R2$ receptor comprising two polypeptides, one of which is a $GABA_BR2$ polypeptide and another of which is a $GABA_BR1$ polypeptide.

This invention is directed to a method of forming a $GABA_BR1/R2$ receptor which comprises inducing cells to express both a $GABA_BR1$ polypeptide and a $GABA_BR2$ polypeptide.

$GABA_BR1$ as used in this application could be $GABA_BR1a$ or $GABA_BR1b$. The observation that at least two variants of the $GABA_BR1$ polypeptide exist raises the possibility that $GABA_BR2$ splice variants may exist or that there may exist introns in coding or non-coding regions of the genes encoding the $GABA_BR2$ polypeptides. In addition, spliced form(s) of mRNA may encode additional amino acids either upstream of the currently defined starting

methionine or within the coding region. Further, the existence and use of alternative exons is possible, whereby the mRNA may encode different amino acids within the region comprising the exon. In addition, single amino acid substitutions may arise via the mechanism of RNA editing such that the amino acid sequence of the expressed protein is different than that encoded by the original gene (Burns et al., 1996; Chu et al., 1996). Such variants may exhibit pharmacologic properties differing from the polypeptide encoded by the original gene.

The activity of a G-protein coupled receptor (GPCR) typically is measured using any of a variety of functional assays in which activation of the receptor in question results in an observable change in the level of some second messenger system, including but not limited to adenylate cyclase, calcium mobilization, arachidonic acid release, ion channel activity, inositol phospholipid hydrolysis or guanylyl cyclase. Heterologous expression systems utilizing appropriate host cells to express the nucleic acids of the subject invention are used to obtain the desired second messenger coupling. Receptor activity may also be assayed in an oocyte expression system.

The pharmacologic properties of the receptor described herein when $GABA_BR2$ is co-expressed with $GABA_BR1$, are similar to the pharmacologic properties of the $GABA_B$ receptor observed using tissues. For convenience, in the context of the present invention applicants will refer to the product of the heterologous coexpression of $GABA_BR2$ and $GABA_BR1$ as the "$GABA_BR1/R2$ receptor." Thus, a cell expressing nucleic acid encoding a $GABA_BR1/R2$ receptor is to be understood to refer to a cell expressing both nucleic acid encoding a $GABA_BR1$ polypeptide and nucleic acid encoding a $GABA_BR2$

polypeptide. In this application, $GABA_BR1$ can be either $GABA_BR1a$ or $GABA_BR1b$.

This invention is directed to an antibody capable of binding to a $GABA_BR1/R2$ receptor, wherein the $GABA_BR2$ polypeptide is encoded by an above-identified nucleic acid.

This invention is directed to an above-identified antibody, wherein the $GABA_BR2$ polypeptide is a human $GABA_BR2$ polypeptide.

This invention is directed to an antibody capable of competitively inhibiting the binding of an above-identified antibody to a $GABA_BR1/R2$ receptor.

In one embodiment, the antibody is a monoclonal antibody.

This invention is directed to an above-identified monoclonal antibody directed to an epitope of a $GABA_BR1/R2$ receptor present on the surface of a $GABA_BR1/R2$ polypeptide expressing cell.

This invention is directed to a pharmaceutical composition which comprises an amount of an above-identified antibody effective to block binding of a ligand to the $GABA_BR1/R2$ receptor and a pharmaceutically acceptable carrier.

This invention is directed to a transgenic, nonhuman mammal expressing a $GABA_BR1/R2$ receptor, which is not naturally expressed by the mammal.

This invention is directed to a transgenic, nonhuman mammal comprising a homologous recombination knockout of the native $GABA_BR1/R2$ receptor.

In one embodiment, the transgenic nonhuman mammal is a mouse.

This invention is directed to a method of detecting the presence of a $GABA_BR1/R2$ receptor on the surface of a cell which comprises contacting the cell with an above-identified antibody under conditions permitting binding of the antibody to the receptor, detecting the presence of the antibody bound to the cell, and thereby detecting the presence of a $GABA_BR1/R2$ receptor on the surface of the cell.

This invention is directed to a method of determining the physiological effects of varying levels of activity of $GABA_BR1/R2$ receptors which comprises producing an above-identified transgenic nonhuman mammal whose levels of $GABA_BR1/R2$ receptor activity vary due to the presence of an inducible promoter which regulates $GABA_BR1/R2$ receptor expression.

This invention is directed to a method of determining the physiological effects of varying levels of activity of $GABA_BR1/R2$ receptors which comprises producing a panel of above-identified transgenic nonhuman mammals, each expressing a different amount of $GABA_BR1/R2$ receptor.

This invention is directed to a method for identifying an antagonist capable of alleviating an abnormality, by decreasing the activity of a $GABA_BR1/R2$ receptor comprising administering a compound to a above-identified transgenic nonhuman mammal, and determining whether the compound alleviates the physical and behavioral abnormalities displayed by the transgenic, nonhuman mammal, the alleviation of the abnormality identifying the compound as the antagonist.

This invention is directed to an antagonist identified by an above-identified method.

This invention is directed to a pharmaceutical composition comprising an above-identified antagonist and a pharmaceutically acceptable carrier.

This invention is directed to a method of treating an abnormality in a subject wherein the abnormality is alleviated by decreasing the activity of a $GABA_BR1/R2$ receptor which comprises administering to a subject an effective amount of an above-identified pharmaceutical composition, thereby treating the abnormality.

This invention is directed to a method for identifying an agonist capable of alleviating an abnormality, by increasing the activity of a $GABA_BR1/R2$ receptor comprising administering a compound to an above-identified transgenic nonhuman mammal, and determining whether the compound alleviates the physical and behavioral abnormalities displayed by the transgenic, nonhuman mammal, the alleviation of the abnormality identifying the compound as the agonist.

This invention is directed to an agonist identified by an above-identified method.

This invention is directed to a pharmaceutical composition comprising an above-identified agonist and a pharmaceutically acceptable carrier.

This invention is directed to a method for treating an abnormality in a subject wherein the abnormality is alleviated by increasing the activity of a $GABA_BR1/R2$ receptor which comprises administering to a subject an effective amount of an above-identified pharmaceutical composition, thereby treating the abnormality.

This invention is directed to a cell which expresses on its surface a mammalian $GABA_BR1/R2$ receptor that is not naturally expressed on the surface of such cell.

This invention is directed to a cell, wherein the mammalian $GABA_BR1/R2$ receptor comprises two polypeptides, one of which is a $GABA_BR2$ polypeptide and another of which is a $GABA_BR1$ polypeptide.

This invention is directed to a process for identifying a chemical compound which specifically binds to a $GABA_BR1/R2$ receptor which comprises contacting cells containing nucleic acid encoding and expressing on their cell surface the $GABA_BR1/R2$ receptor, wherein such cells do not normally express the $GABA_BR1/R2$ receptor, with the compound under conditions suitable for binding, and detecting specific binding of the chemical compound to the $GABA_BR1/R2$ receptor.

This invention is directed to a process for identifying a chemical compound which specifically binds to a $GABA_BR1/R2$ receptor which comprises contacting a membrane fraction from a cell extract of cells containing nucleic acid encoding and expressing on their cell surface the $GABA_BR1/R2$ receptor, wherein such cells do not normally express the $GABA_BR1/R2$ receptor, with the compound under conditions suitable for binding, and detecting specific binding of the chemical compound to the $GABA_BR1/R2$ receptor.

In one embodiment, the $GABA_BR1/R2$ receptor is a mammalian $GABA_BR1/R2$ receptor.

In another embodiment, the $GABA_BR1/R2$ receptor comprises a $GABA_BR2$ polypeptide which has substantially the same amino acid sequence as that encoded by the plasmid BO-55 (ATCC Accession No. 209104).

In another embodiment, the $GABA_BR1/R2$ receptor comprises a $GABA_BR2$ polypeptide which has substantially the same sequence as the amino acid sequence shown in Figures 23A-23D (Seq. ID No. 47).

In another embodiment, the $GABA_BR1/R2$ receptor comprises a $GABA_BR2$ polypeptide which has the amino acid sequence shown in Figures 23A-23D (Seq. ID No. 47).

In another embodiment, the $GABA_BR1/R2$ receptor comprises a $GABA_BR2$ polypeptide which has substantially the same amino acid sequence as that encoded by the plasmid pEXJT3T7-hGABAB2 (ATCC Accession No. 203515).

In another embodiment, the $GABA_BR1/R2$ receptor comprises a $GABA_BR2$ polypeptide which has substantially the same amino acid sequence as the sequence shown in Figures 23A-23D (Seq. ID No. 47).

In another embodiment, the $GABA_BR1/R2$ receptor comprises a $GABA_BR2$ polypeptide which has the sequence shown in Figures 23A-23D (Seq. ID No. 47).

In another embodiment, the compound is not previously known to bind to a $GABA_BR1/R2$ receptor.

This invention is directed to a compound identified by an above-identified process.

In one embodiment, the cell is an insect cell.

In another embodiment, the cell is a mammalian cell.

In another embodiment, the cell is nonneuronal in origin.

In another embodiment, the nonneuronal cell is a COS-7

cell, 293 human embryonic kidney cell, a CHO cell, a NIH-3T3 cell a mouse Y1 cell or LM(tk-) cell.

In another embodiment, the compound is not previously known to bind to a $GABA_BR1/R2$ receptor.

This invention is directed to a compound identified by an above-identified process.

This invention is directed to a process involving competitive binding for identifying a chemical compound which specifically binds to a $GABA_BR1/R2$ receptor which comprises separately contacting cells expressing on their cell surface the $GABA_BR1/R2$ receptor, wherein such cells do not normally express the $GABA_BR1/R2$ receptor, with both the chemical compound and a second chemical compound known to bind to the receptor, and with only the second chemical compound, under conditions suitable for binding of both compounds, and detecting specific binding of the chemical compound to the $GABA_BR1/R2$ receptor, a decrease in the binding of the second chemical compound to the $GABA_BR1/R2$ receptor in the presence of the chemical compound indicating that the chemical compound binds to the $GABA_BR1/R2$ receptor.

This invention is directed to a process involving competitive binding for identifying a chemical compound which specifically binds to a human $GABA_BR1/R2$ receptor which comprises separately contacting a membrane fraction from a cell extract of cells expressing on their cell surface the $GABA_BR1/R2$ receptor, wherein such cells do not normally express the $GABA_BR1/R2$ receptor, with both the chemical compound and a second chemical compound known to bind to the receptor, and with only the second chemical compound, under conditions suitable for binding of both compounds, and detecting specific binding of the chemical compound to the $GABA_BR1/R2$

receptor, a decrease in the binding of the second chemical compound to the $GABA_BR1/R2$ receptor in the presence of the chemical compound indicating that the chemical compound binds to the $GABA_BR1/R2$ receptor.

In one embodiment, the $GABA_BR1/R2$ receptor is a mammalian $GABA_BR1/R2$ receptor.

In another embodiment, the $GABA_BR1/R2$ receptor comprises a $GABA_BR2$ polypeptide which has substantially the same amino acid sequence as that encoded by plasmid BO-55 (ATCC Accession No. 209104).

In another embodiment, the $GABA_BR1/R2$ receptor comprises a $GABA_BR2$ polypeptide which has substantially the same amino acid sequence as that shown in Figures 23A-23D (Seq. ID No. 47).

In another embodiment, the $GABA_BR1/R2$ receptor comprises a $GABA_BR2$ polypeptide which has the amino acid sequence shown in Figures 23A-23D (Seq. ID No. 47).

In another embodiment, the $GABA_BR1/R2$ receptor comprises a $GABA_BR2$ polypeptide which has substantially the same amino acid sequence as that encoded by plasmid pEXJT3T7-hGABAB2 (ATCC Accession No. 203515).

In another embodiment, the $GABA_BR1/R2$ receptor comprises a $GABA_BR2$ polypeptide which has substantially the same amino acid sequence as the sequence shown in Figures 23A-23D (Seq. ID No. 47).

In another embodiment, the $GABA_BR1/R2$ receptor comprises a $GABA_BR2$ polypeptide which has the sequence shown in Figures 23A-23D (Seq. ID No. 47).

In another embodiment, the cell is an insect cell.

In another embodiment, the cell is a mammalian cell.

In another embodiment, the cell is nonneuronal in origin.

In another embodiment, the nonneuronal cell is a COS-7 cell, 293 human embryonic kidney cell, a CHO cell, a NIH-3T3 cell a mouse Y1 cell or LM(tk-) cell.

In another embodiment, the compound is not previously known to bind to a $GABA_BR1/R2$ receptor.

This invention is directed to a compound identified by an above-identified process.

This invention is directed to a method of screening a plurality of chemical compounds not known to bind to a $GABA_BR1/R2$ receptor to identify a compound which specifically binds to the $GABA_BR1/R2$ receptor, which comprises

(a) contacting cells containing nucleic acid encoding and expressing on their cell surface the $GABA_BR1/R2$ receptor, wherein such cells do not normally express the $GABA_BR1/R2$ receptor, with a compound known to bind specifically to the $GABA_BR1/R2$ receptor;

(b) contacting the same cells as in step (a) with the plurality of compounds not known to bind specifically to the $GABA_BR1/R2$ receptor, under conditions permitting binding of compounds known to bind the $GABA_BR1/R2$ receptor;

(c) determining whether the binding of the compound known to bind specifically to the $GABA_BR1/R2$ receptor is reduced in the presence

of the plurality of the compounds, relative to the binding of the compound in the absence of the plurality of compounds, and if the binding is reduced;

(d) separately determining the extent of binding to the $GABA_BR1/R2$ receptor of each compound included in the plurality of compounds, so as to thereby identify the compound or compounds present in such plurality of compounds which specifically binds to the $GABA_BR1/R2$ receptor.

This invention is directed to a method of screening a plurality of chemical compounds not known to bind to a $GABA_BR1/R2$ receptor to identify a compound which specifically binds to the $GABA_BR1/R2$ receptor, which comprises

(a) contacting a membrane fraction extract from cells containing nucleic acid encoding and expressing on their cell surface the $GABA_BR1/R2$ receptor, wherein such cells do not normally express the $GABA_BR1/R2$ receptor, with a compound known to bind specifically to the $GABA_BR1/R2$ receptor;

(b) contacting the same membrane fraction as in step (a) with the plurality of compounds not known to bind specifically to the $GABA_BR1/R2$ receptor, under conditions permitting binding of compounds known to bind the $GABA_BR1/R2$ receptor;

(c) determining whether the binding of the compound known to bind specifically to the $GABA_BR1/R2$ receptor is reduced in the presence of the plurality of compounds, relative to

the binding of the compound in the absence of the plurality of compounds, and if the binding is reduced;

(d) separately determining the extent of binding to the $GABA_BR1/R2$ receptor of each compound included in the plurality of compounds, so as to thereby identify the compound or compounds present in such plurality of compounds which specifically binds to the $GABA_BR1/R2$ receptor.

In one embodiment, the $GABA_BR1/R2$ receptor is a mammalian $GABA_BR1/R2$ receptor.

In one embodiment, the cell is a mammalian cell.

In one embodiment, the mammalian cell is non-neuronal in origin.

In one embodiment, the non-neuronal cell is a COS-7 cell, a 293 human embryonic kidney cell, a LM(tk-) cell, a CHO cell, a mouse Y1 cell or an NIH-3T3 cell.

This invention is directed to a process for determining whether a chemical compound is a $GABA_BR1/R2$ receptor agonist which comprises contacting cells with the compound under conditions permitting the activation of the $GABA_BR1/R2$ receptor, and detecting an increase in $GABA_BR1/R2$ receptor activity, so as to thereby determine whether the compound is a $GABA_BR1/R2$ receptor agonist.

This invention is directed to a process for determining whether a chemical compound is a $GABA_BR1/R2$ receptor antagonist which comprises contacting cells containing nucleic acid encoding and expressing on their cell surface the $GABA_BR1/R2$ receptor, wherein such cells do not normally express the $GABA_BR1/R2$ receptor, with the

compound in the presence of a known $GABA_BR1/R2$ receptor agonist, under conditions permitting the activation of the $GABA_BR1/R2$ receptor, and detecting a decrease in $GABA_BR1/R2$ receptor activity, so as to thereby determine whether the compound is a $GABA_BR1/R2$ receptor antagonist.

Expression of genes in Xenopus oocytes is well known in the art (A. Coleman, Transcription and Translation: A Practical Approach (B.D. Hanes, S.J. Higgins, eds., pp 271-302, IRL Press, Oxford, 1984; Y. Masu et al., Nature **329**:21583-21586, 1994) and is performed using microinjection of native mRNA or in vitro synthesized mRNA into frog oocytes. The preparation of in vitro synthesized mRNA can be performed by various standard techniques (J. Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989) including using T7 polymerase with the mCAP RNA capping kit (Stratagene).

In one embodiment, the cells additionally express nucleic acid encoding GIRK1 and GIRK4.

In another embodiment, the $GABA_BR2$ receptor is a mammalian $GABA_BR2$ receptor.

This invention is directd to a pharmaceutical composition which comprises an amount of a $GABA_BR1/R2$ receptor agonist determined to be an agonist by an above-identified process effective to increase activity of a $GABA_BR1/R2$ receptor and a pharmaceutically acceptable carrier.

This invention is directed to a pharmaceutical, wherein the $GABA_BR1/R2$ receptor agonist was not previously known.

This invention is directed to a pharmaceutical composition which comprises an amount of a $GABA_BR1/R2$ receptor antagonist determined to be an antagonist an above-identified process effective to reduce activity of a $GABA_BR1/R2$ receptor and a pharmaceutically acceptable carrier.

This invention is directed to a pharmaceutical composition, wherein the $GABA_BR1/R2$ receptor antagonist was not previously known.

This invention is directed to a process for determining whether a chemical compound activates a $GABA_BR1/R2$ receptor, which comprises contacting cells producing a second messenger response and expressing on their cell surface the $GABA_BR1/R2$ receptor, wherein such cells do not normally express the $GABA_BR1/R2$ receptor, with the chemical compound under conditions suitable for activation of the $GABA_BR1/R2$ receptor, and measuring the second messenger response in the presence and in the absence of the chemical compound, a change in the second messenger response in the presence of the chemical compound indicating that the compound activates the $GABA_BR1/R2$ receptor.

In one embodiment, the second messenger response comprises potassium channel activation and the change in second messenger is an increase in the level of potassium current.

This invention is directed to a process for determining whether a chemical compound inhibits activation of a $GABA_BR1/R2$ receptor, which comprises separately contacting cells producing a second messenger response and expressing on their cell surface the $GABA_BR1/R2$ receptor, wherein such cells do not normally express the $GABA_BR1/R2$ receptor, with both the

chemical compound and a second chemical compound known to activate the $GABA_BR1/R2$ receptor, and with only the second chemical compound, under conditions suitable for activation of the $GABA_BR1/R2$ receptor, and measuring the second messenger response in the presence of only the second chemical compound and in the presence of both the second chemical compound and the chemical compound, a smaller change in the second messenger response in the presence of both the chemical compound and the second chemical compound than in the presence of only the second chemical compound indicating that the chemical compound inhibits activation of the $GABA_BR1/R2$ receptor.

In one embodiment, the second messenger response comprises potassium channel activation and the change in second messenger response is a smaller increase in the level of inward potassium current in the presence of both the chemical compound and the second chemical compound than in the presence of only the second chemical compound.

This invention is directed to an above-identified process, wherein the $GABA_BR1/R2$ receptor is a mammalian $GABA_BR1/R2$ receptor.

In one embodiment, the $GABA_BR1/R2$ receptor comprises a $GABA_BR2$ polypeptide which has substantially the same amino acid sequence as that encoded by the plasmid BO-55 (ATCC Accession No. 209104).

In another embodiment, the $GABA_BR1/R2$ receptor comprises a $GABA_BR2$ polypeptide which has substantially the same amino acid sequence as that shown in Figures 4A-4D (Seq. ID No. 4).

In another embodiment, the $GABA_BR1/R2$ receptor comprises

a $GABA_BR2$ polypeptide which has substantially the same amino acid sequence as that shown in Figures 23A-23D (Seq. ID No. 47).

In another embodiment, the $GABA_BR1/R2$ receptor comprises a $GABA_BR2$ polypeptide which has the sequence, shown in Figures 23A-23D (Seq. ID No. 47).

In another embodiment, the $GABA_BR1/R2$ receptor comprises a $GABA_BR2$ polypeptide which has substantially the same amino acid sequence as that encoded by the plasmid pEXJT3T7-hGABAB2 (ATCC Accession No. 203515).

This invention is directed to an above-identified process, wherein the cell is an insect cell.

This invention is directed to an above-identified process, wherein the cell is a mammalian cell.

In one embodiment, the mammalian cell is nonneuronal in origin.

In another embodiment, the nonneuronal cell is a COS-7 cell, CHO cell, 293 human embryonic kidney cell, NIH-3T3 cell or LM(tk-) cell.

In another embodiment, the compound was not previously known to activate or inhibit a $GABA_BR1/R2$ receptor.

This invention is directed to a compound determined by an above-identified process.

This invention is directed to a pharmaceutical composition which comprises an amount of a $GABA_BR1/R2$ receptor agonist determined by an above-identified process effective to increase activity of a $GABA_BR1/R2$ receptor and a pharmaceutically acceptable carrier.

In one embodiment, the $GABA_BR1/R2$ receptor agonist was not previously known.

This invention is directed to a pharmaceutical composition which comprises an amount of a $GABA_BR1/R2$ receptor antagonist determined by an above-identified process effective to reduce activity of a $GABA_BR1/R2$ receptor and a pharmaceutically acceptable carrier.

In one embodiment, the $GABA_BR1/R2$ receptor antagonist was not previously known.

This invention is directed to method of screening a plurality of chemical compounds not known to activate a $GABA_BR1/R2$ receptor to identify a compound which activates the $GABA_BR1/R2$ receptor which comprises:

(a) contacting cells containing nucleic acid encoding and expressing on their cell surface the $GABA_BR1/R2$ receptor, wherein such cells do not normally express the $GABA_BR1/R2$ receptor, with the plurality of compounds not known to activate the $GABA_BR1/R2$ receptor, under conditions permitting activation of the $GABA_BR1/R2$ receptor;

(b) determining whether the activity of the $GABA_BR1/R2$ receptor is increased in the presence of the compounds, and if it is increased;

(c) separately determining whether the activation of the $GABA_BR1/R2$ receptor is increased by each compound included in the plurality of compounds, so as to thereby identify the compound or compounds present in such plurality of compounds which activates the

$GABA_BR1/R2$ receptor.

In one embodiment, the cells express nucleic acid encoding GIRK1 and GIRK4.

In another embodiment, the $GABA_BR1/R2$ receptor is a mammalian $GABA_BR1/R2$ receptor.

This invention is directed to a method of screening a plurality of chemical compounds not known to inhibit the activation of a $GABA_BR1/R2$ receptor to identify a compound which inhibits the activation of the $GABA_BR1/R2$ receptor, which comprises:

(a) contacting cells containing nucleic acid encoding and expressing on their cell surface the $GABA_BR1/R2$ receptor, wherein such cells do not normally express the $GABA_BR1/R2$ receptor, with the plurality of compounds in the presence of a known $GABA_BR1/R2$ receptor agonist, under conditions permitting activation of the $GABA_BR1/R2$ receptor;

(b) determining whether the activation of the $GABA_BR1/R2$ receptor is reduced in the presence of the plurality of compounds, relative to the activation of the $GABA_BR1/R2$ receptor in the absence of the plurality of compounds, and if it is reduced;

(c) separately determining the inhibition of activation of the $GABA_BR1/R2$ receptor for each compound included in the plurality of compounds, so as to thereby identify the compound or compounds present in such a plurality of compounds which inhibits the activation of the $GABA_BR1/R2$ receptor.

In one embodiment, the cells express nucleic acid encoding GIRK1 and GIRK4.

In one embodiment, the $GABA_BR1/R2$ receptor is a mammalian $GABA_BR1/R2$ receptor.

In another embodiment, wherein the cell is a mammalian cell.

In another embodiment, the mammalian cell is non-neuronal in origin.

In another embodiment, the non-neuronal cell is a COS-7 cell, a 293 human embryonic kidney cell, a LM(tk-) cell or an NIH-3T3 cell.

This invention is directed to a pharmaceutical composition comprising a compound identified by an above-identified method, effective to increase $GABA_BR1/R2$ receptor activity and a pharmaceutically acceptable carrier.

This invention is directed to a pharmaceutical composition comprising a compound identified by an above-identified method, effective to decrease $GABA_BR1/R2$ receptor activity and a pharmaceutically acceptable carrier.

This invention is directed to a process for determining whether a chemical compound is a $GABA_BR1/R2$ receptor agonist, which comprises preparing a membrane fraction from cells which comprise nucleic acid encoding and expressing on their cell surface the $GABA_BR1/R2$ receptor, wherein such cells do not normally express the $GABA_BR1/R2$ receptor, separately contacting the membrane fraction with both the chemical compound and GTPγS, and with only GTPγS, under conditions permitting

the activation of the $GABA_BR1/R2$ receptor, and detecting GTPγS binding to the membrane fraction, an increase in GTPγS binding in the presence of the compound indicating that the chemical compound activates the $GABA_BR1/R2$ receptor.

This invention is directed to a process for determining whether a chemical compound is a $GABA_BR1/R2$ receptor antagonist, which comprises preparing a membrane fraction from cells which comprise nucleic acid encoding and expressing on their cell surface the $GABA_BR1/R2$ receptor, wherein such cells do not normally express the $GABA_BR1/R2$ receptor, separately contacting the membrane fraction with the chemical compound, GTPγS and a second chemical compound known to activate the $GABA_BR1/R2$ receptor, with GTPγS and only the second compound, and with GTPγS alone, under conditions permitting the activation of the $GABA_BR1/R2$ receptor, detecting GTPγS binding to each membrane fraction, and comparing the increase in GTPγS binding in the presence of the compound and the second compound relative to the binding of GTPγS alone, to the increase in GTPγS binding in the presence of the second chemical compound known to activate the $GABA_BR1/R2$ receptor relative to the binding of GTPγS alone, a smaller increase in GTPγS binding in the presence of the compound and the second compound indicating that the compound is a $GABA_BR1/R2$ receptor antagonist.

In one embodiment, the $GABA_BR2$ receptor is a mammalian $GABA_BR2$ receptor.

In another embodiment, the $GABA_BR1/R2$ receptor comprises a $GABA_BR2$ polypeptide which has substantially the same amino acid sequence as that encoded by the plasmid BO-55 (ATCC Accession No. 209104).

In another embodiment, the $GABA_B$R1/R2 receptor comprises a $GABA_B$R2 polypeptide which has substantially the same amino acid sequence as that shown in Figures 4A-4D (Seq. ID No. 4).

In another embodiment, the $GABA_B$R1/R2 receptor comprises a $GABA_B$R2 polypeptide which has substantially the same amino acid sequence as that encoded by the plasmid pEXJT3T7-hGABAB2 (ATCC Accession No. 203515).

In another embodiment, the $GABA_B$R1/R2 receptor comprises a $GABA_B$R2 polypeptide which has substantially the same amino acid sequence as that shown in Figures 23A-23D (Seq. ID No. 47).

In another embodiment, the $GABA_B$R1/R2 receptor comprises a $GABA_B$R2 polypeptide which has the sequence shown in Figures 23A-23D (Seq. ID No. 47).

In another embodiment, the cell is an insect cell.

In another embodiment, the cell is a mammalian cell.

In another embodiment, the mammalian cell is nonneuronal in origin.

In another embodiment, the nonneuronal cell is a COS-7 cell, CHO cell, 293 human embryonic kidney cell, NIH-3T3 cell or LM(tk-) cell.

In another embodiment, the compound was not previously known to be an agonist or antagonist of a $GABA_B$R1/R2 receptor.

This invention is directed to a compound determined to be an agonist or antagonist of a $GABA_B$R1/R2 receptor by an above-identified process.

This invention is directed to a method of treating spasticity in a subject which comprises administering to the subject an amount of a compound which is an agonist of a $GABA_BR1/R2$ receptor effective to treat spasticity in the subject.

This invention is directed to a method of treating asthma in a subject which comprises administering to the subject an amount of a compound which is a $GABA_BR1/R2$ receptor agonist effective to treat asthma in the subject.

This invention is directed to a method of treating incontinence in a subject which comprises administering to the subject an amount of a compound which is a $GABA_BR1/R2$ receptor agonist effective to treat incontinence in the subject.

This invention is directed to method of decreasing nociception in a subject which comprises administering to the subject an amount of a compound which is a $GABA_BR1/R2$ receptor agonist effective to decrease nociception in the subject.

This invention is directed to a use of a $GABA_BR2$ agonist as an antitussive agent which comprises administering to the subject an amount of a compound which is a $GABA_BR1/R2$ receptor agonist effective as an antitussive agent in the subject.

This invention is directed to a method of treating drug addiction in a subject which comprises administering to the subject an amount of a compound which is a $GABA_BR1/R2$ receptor agonist effective to treat drug addiction in the subject.

This invention directed to a method of treating

Alzheimer's disease in a subject which comprises administering to the subject an amount of a compound which is a $GABA_BR1/R2$ receptor antagonist effective to treat Alzheimer's disease in the subject.

This invention is directed to a peptide selected from the group consisting of:

a) P L Y S I L S A L T I L G M I M A S A F L F F N I K N; (SEQ ID NO:48)

b) L I I L G G M L S Y A S I F L F G L D G S F V S E K T; (SEQ ID NO:49)

c) C T V R T W I L T V G Y T T A F G A M F A K T W R; (SEQ ID NO:50)

d) Q K L L V I V G G M L L I D L C I L I C W Q; (SEQ ID NO:51)

e) M T I W L G I V Y A Y K G L L M L F G C F L A W; (SEQ ID NO:52)

f) A L N D S K Y I G M S V Y N V G I M C I I G A A V; and (SEQ ID NO:53)

g) C I V A L V I I F C S T I T L C L V F V P K L I T L R T N. (SEQ ID NO:54)

This invention is directed to a compound that prevents the formation of a $GABA_BR1/R2$ receptor complex.

Transmembrane peptides derived from $GABA_BR2$ sequences may modulate the functional activity of $GABA_BR1/R2$ receptors. One mode of action involves the destruction of the $GABA_BR1/R2$ receptor complex *via* competitive displacement of the $GABA_BR2$ polypeptide subunit by the peptide upon binding to the $GABA_BR1$ polypeptide subunit. The peptides may be synthesized using standard solid phase F-moc peptide synthesis protocol using an Advanced Chemtech 396 Automated Peptide Synthesizer.

Additional $GABA_B$ subtypes in hypothalamus and caudate putamen are predicted due to the under-representation of $GABA_BR2$ hybridization signals. These novel $GABA_B$

proteins and others may be identified by using $GABA_BR2$ polypeptides in co-immunoprecipitation experiments.

This invention provides a process for making a composition of matter which specifically binds to a $GABA_BR1/R2$ receptor which comprises identifying a chemical compound using any of the processes descirbed herein for identifying a compound which binds to and/or activates or inhibits activation of a $GABA_BR1/R2$ receptor and then synthesizing the chemical compound or a novel structural and functional analog or homolog thereof. In one embodiment, the $GABA_BR1/R2$ receptor is a human $GABA_BR1/R2$ receptor.

This invention further provides a process for preparing a pharmaceutical composition which comprises admixing a pharmaceutically acceptable carrier and a pharmaceutically acceptable amount of a chemical compound identified by any of the processes described herein for identifying a compound which binds to and/or activates or inhibits activation of a $GABA_BR1/R2$ receptor or a novel structural and functional analog or homolog thereof. In one embodiment, the $GABA_BR1/R2$ receptor is a human $GABA_BR1/R2$ receptor.

Thus, once the gene for a targeted receptor subtype is cloned, it is placed into a recipient cell which then expressses the targeted receptor subtype on its surface. This cell, which expresses a single population of the targeted human receptor subtype, is then propagated resulting in the establishment of a cell line. This cell line, which constitutes a drug discovery system, is used in two different types of assays: binding assays and functional assays. In binding assays, the affinity of a compound for both the receptor subtype that is the target of a particular drug discovery program and other receptor subtypes that

could be associated with side effects are measured. These measurements enable one to predict the potency of a compound, as well as the degree of selectivity that the compound has for the targeted receptor subtype over other receptor subtypes. The data obtained from binding assays also enable chemists to design compounds toward or away from one or more of the relevant subtypes, as appropriate, for optimal therapeutic efficacy. In functional assays, the nature of the response of the receptor subtype to the compound is determined. Data from the functional assays show whether the compound is acting to inhibit or enhance the activity of the receptor subtype, thus enabling pharmacologists to evaluate compounds rapidly at their ultimate human receptor subtypes targets permitting chemists to rationally design drugs that will be more effective and have fewer or substantially less severe side effects than existing drugs.

Approaches to designing and synthesizing receptor subtype-selective compounds are well known and include traditional medicinal chemistry and the newer technology of combinatorial chemistry, both of which are supported by computer-assisted molecular modeling. With such approaches, chemists and pharmacologists use their knowledge of the structures of the targeted receptor subtype and compounds determined to bind and/or activate or inhibit activation of the receptor subtype to design and synthesize structures that will have activity at these receptor subtypes.

Combinatorial chemistry involves automated synthesis of a variety of novel compounds by assembling them using different combinations of chemical building blocks. The use of combinatorial chemistry greatly accelerates the process of generating compounds. The resulting arrays of compounds are called libraries and are used to

screen for compounds (lead compounds) that demonstrate a sufficient level of activity at receptors of interest. Using combinatorial chemistry it is possible to synthesize focused libraries of compounds anticiapted to be highly biased toward the receptor target of interest.

Once lead compounds are identified, whether through the use of combinatorial chemistry or traditional medicinal chemistry or otherwise, a variety of homologs and analogs are prepared to facilitate an understanding of the relationship between chemical structure and biological or functional activity. These studies define structure activity relationships which are then used to design drugs with improved potency, selectivity and pharmacokinetic properties. Combinatorial chemistry is also used to rapidly generate a variety of structures for lead optimization. Traditional medicinal chemistry, which involves the synthesis of compounds one at a time, is also used for further refinement and to generate compounds not accessible by autometed techniques. Once such drugs are defined the production is scaled up using standard chemical manufacturing methodiologies utilized throughout the pharmaceutical and chemistry industry.

This invention will be better understood from the Experimental Details which follow. However, one skilled in the art will readily appreciate that the specific methods and results discussed are merely illustrative of the invention as described more fully in the claims which follow thereafter.

Experimental Details

Materials and Methods

DNA Sequencing

DNA sequences were determined using an ABI PRISM 377 DNA Sequencer (Perkin-Elmer, Foster City, CA) according to the manufacturer's instructions.

Hybridization methodology

Probes were end-labeled with polynucleotide kinase according to the manufacturer's instructions (Boehringer-Mannheim). Hybridization was performed on Zeta-Probe membrane (Bio-Rad, CA) at reduced stringency: 40°C in a solution containing 25% formamide, 5x SSC (1x SSC = 0.15 M NaCl, 0.015 M sodium citrate), 1x Denhardt's solution (0.02% polyvinylpyrrolindone, 0.02% Ficoll, 0.02% bovine serum albumin) and 25 μg/μL sonicated salmon sperm DNA. Membrane strips were washed at 40°C in 0.1x SSC containing 0.1% SDS and exposed at -70°C to Kodak XAR film in the presence of an intensifying screen.

The nucleotide sequences of the hybridization probes are shown below:

T-891: 5'-AGGGATGCTTTCCTATGCTTCCATATTTCTCTTTGGCCTTGATGG-3' (Seq. ID No. 5) Nucleotides 1449-1493 of TL-267, forward strand.

T-892: 5'-CAATGTGCAGTTCTGCATCGTGGCTCTGGTCATCATCTTCTGCAG-3' (Seq. ID No. 6) Nucleotides 2022-2066 of TL-267, forward strand.

PCR Methodology

PCR reactions were carried out using a PE 9600 (Perkin-Elmer) PCR cycler in 20 μL volumes using Expand Long

Template Polymerase (Boehringer-Mannheim) and the manufacturer's buffer 1 for internal PCR primers or manufacturer's buffer 2 for vector-anchored PCR. Reactions were run using a program consisting of 35 cycles of 94°C for 30 sec., 68°C for 20 sec, and 72°C for 1 min, with a pre-incubation at 95°C for 5 min and post-incubation hold at 4°C.

Nucleotide sequences of the primer sets used in PCR reactions are shown below:

T-94: 5'-CTTCTAGGCCTGTACGGAAGTGTT-3' (Seq. ID No. 7); vector, forward primer.

T-95: 5'-GTTGTGGTTTGTCCAAACTCATCAAT-3' (Seq. ID No. 8); vector, reverse primer.

T-887: 5'-GGGATGAGTGTCTACAACGTGGGG-3' (Seq. ID No. 9); nucleotides 1948-1971 of TL-267, forward primer.

T-888: 5'-TGCGTTGCTGCATCTGGGTTTGTTCT-3' (Seq. ID No. 10); nucleotides 2138-2113 of TL-267, reverse primer.

T-889: 5'-ATCTCCCTACCTCTCTACAGCATCCT-3' (Seq. ID No. 11); nucleotides 1300-1325 of TL-267, forward primer.

T-890: 5'-CAGGTCCTGACGGTGCAAAGTGTTTC-3' (Seq. ID No. 12); nucleotides 1544-1519 of TL-267, reverse primer.

T-921: 5'-TGACGCAAGACGTTCAGAGGTTCTCT-3' (Seq. ID No. 13); nucleotides 473-498 of TL-267, forward primer.

T-922: 5'-TGTAGCCTTCCATGGCAGCAAGCAGA-3' (Seq. ID No. 14); nucleotides 814-789 of TL-267, reverse primer.

T-923: 5'-AGAGAACCTCTGAACGTCTTGCGTCA-3' (Seq. ID No. 15); nucleotides 498-473 of TL-267, reverse primer.

T-935: 5'-GGCTCTGTTGTGTTCCACTGTAGCTG-3' (Seq. ID No. 16); nucleotides 2483-2458 of TL-267, reverse primer.

T-938: 5'-TCATGCCGCTCACCAAGGAGGTGGCC-3' (Seq. ID No. 17); nucleotides 53 to 78 of TL-267, forward primer.

T-939: 5'-GGCCACCTCCTTGGTGAGCGGCATGA-3' (Seq. ID No. 18); nucleotides 78 to 53 of TL-267, reverse primer.

T-947: 5'-TGAGTGAGCAGAGTCCAGAGCCGT-3' (Seq. ID No. 19); nucleotides -68 to -45 of TL-267, forward primer.

T-948: 5'-ATGGATGGGAGGTAGGCGTGGTGGAG-3' (Seq. ID No. 20); nucleotides 2591-2566 of TL-267, reverse primer.

Preparation of human hippocampal cDNA library

Total RNA was prepared by a modification of the guanidine thiocyanate method, from 6 grams of human hippocampus. Poly A^+RNA was purified with a FastTrack kit (Invitrogen Corp., San Diego, CA). Double stranded (ds) cDNA was synthesized from 4 μg of poly A^+ RNA according to Gübler and Hoffman (1983), except that ligase was omitted in the second strand cDNA synthesis. The resulting DS cDNA was ligated to BstxI/EcoRI adaptors (Invitrogen Corp.), the excess of adaptors was removed by exclusion chromatography. High molecular weight fractions were ligated in pcEXV.BS (An Okayama and Berg expression vector) cut by BstxI as described by Aruffo and Seed (1987). The ligated DNA was electroporated in E. coli MC 1061 (Gene Pulser, Biorad). A total of 2.2 x 10^6 independent clones with an insert mean size of approximately 3 kb was generated. The library was plated on Petri dishes (Ampicillin selection) in pools of 0.4 to 1.2 x 10^4 independent clones. After 18 hours amplification, the bacteria from each pool were scraped, resuspended in 4 mL of LB media and 1.5 mL processed for plasmid

purification by the alkali method (Sambrook et al, 1989). 1 mL aliquots of each bacterial pool were stored at -85°C in 20% glycerol.

BLAST Search that Identified a Novel 7-TM protein Sequence

Sequence analysis was performed with the Wisconsin Package Version 9.0, Genetics Computer Group (GCG), Madison, Wisconsin. The rat $GABA_BR1a$ amino acid sequence (Kaupmann et al. (1997) Nature 386:239) was used as a query to search the EST division of GenBank with BLAST. Two entries, T07621 and Z43654, had probability scores that suggested significant amino acid homology to the $GABA_BR1a$ polypeptide. T07621 had sequence homology from the beginning of the first transmembrane domain to the beginning of third transmembrane domain of the $GABA_BR1a$ polypeptide. Z43654 had sequence homology from the sixth transmembrane domain to the seventh transmembrane domain of the $GABA_BR1a$ polypeptide. The sequence documentation for T07621 and Z43654 was retrieved with Entrez (NCBI) and neither sequence was annotated as having homology to any 7-TM spanning protein.

T07621 and Z43654 are part of the same sequence.

A series of PCR reactions were carried out on human hippocampus DNA with multiple primer sets: primer set T-887/T-888 designed to Z43654 sequence; primer set T-889/T-890 designed to the T07621 sequence; and primer set T-889/T-888 designed to the forward strand of T07621 and the reverse stand of Z43654. The PCR products was loaded on duplicate lanes of an agarose gel and the DNA was southern blotted to a Zeta-Probe membrane (Bio-Rad, CA). The regions of the membrane corresponding to the individual lanes on the gel were cut to produce membrane strips that contained duplicate samples of the DNA. One set of membrane strips was

hybridized with T-891, a probe specific for the T07621 sequence. Another set of membranes was hybridized with T-892, a probe specific to the Z43654 sequence. The membrane from primer set T-887/T-888 hybridized with probe T-892 for the Z43654 sequence. The membrane from primer set T-889/T-890 hybridized with probe T-891 for the T07621 sequence. The membrane from primer set T889/T-888 hybridized with both the T-891 and T-892 probes.

Isolating the full-length human cDNA by PCR Sib Selection.

PCR reactions were carried out on bacterial pools containing a human hippocampus cDNA library. Primer set T-888/T-889 was used to identify the bacterial pools that contained a portion of the novel receptor. Vector-anchored PCR was carried out on the positive pools to determine which pool contained the longest cDNA insert. Four primer sets were used for the vector-anchored PCR: T-94/T-888, T-94/T889, T-95/T888, and T-95/T889. Pool 365 was identified having the longest cDNA inset and the plasmid was sib selected (McCormick, 1987). The nucleotide sequence of clone 365-9-7-4, designated TL-260, was translated into amino acids and compared to the amino acid sequence of the rat $GABA_BR1a$ polypeptide. Relative the rat $GABA_BR1a$ amino acid sequence, TL-260 was truncated at the amino terminus.

A set of PCR primers (T-921/T-922) was made to the 5' region of TL-260 and was used to re-screen the bacterial pools of the human hippocampus library for the missing segment of the novel clone. Vector-anchored PCR was carried out on the positive pools to determine which pool contained the longest cDNA insert. Four primer sets were used for the vector-anchored PCR: T-94/T-921, T-94/T922, T-95/T921, and T-95/T-922. Pool

299 contained the most 5' sequence. A PCR product derived from the primer set T-94/T-923 was isolated (T-261) and sequenced. The putative amino acids derived from TL-261 were compared to the rat $GABA_BR1$ sequence. TL-261 contained an initiation codon but didn't contain a stop codon upstream of the initiation codon.

A set of PCR primers (T-938/T-935) was made to the 5' region of TL-261 and was used to re-screen the bacterial pools of the human hippocampus library for additional sequence. Vector-anchored PCR was carried out on the positive pools to determine which pool contained the longest cDNA insert. Four primer sets were used for the vector-anchored PCR: T-94/T-938, T-94/T939, T-95/T938, and T-95/T-939. A PCR product derived from primer set T-95/T-939 was isolated (T-261a) and sequenced. The putative amino acids derived from T-261a were compared to the rat GABA-1 amino acid sequence. T-261a contained an initiation codon and an in-frame upstream stop codon.

From the vector-anchored PCR, pool 389 contained the longest cDNA insert. This pool was sib selected with the primer set T-947/T-935. The resulting plasmid, 389-20-29-2, was designated TL-266 and was sequenced.

<u>Construction of $GABA_BR2$ polypeptide in expression vector</u>

A Cla-I-Xba-I fragment from TL-266 was subcloned into the expression vector pEXJ.HRT3T7 and designated TL-267. This plasmid (TL-267) was deposited on June 10, 1997, with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A. under the provisions of the Budapest Treaty for the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure and was accorded ATCC Accession No. 209103.

Generation of rat $GABA_BR2$ PCR product

cDNA from rat hippocampus and rat cerebellum were amplified in 50μL PCR reaction mixtures using the Expand Long Template PCR System (as supplied and described by the manufacturer, Boehringer Mannheim) using a program consisting of 40 cycles of 94°C for 1 min, 50°C for 2 min, and 68°C for 2 min, with a pre- and post-incubation of 95°C for 5 min and 68°C for 7 min, respectively. PCR primers for rat $GABA_BR2$ were designed against the human $GABA_BR2$ sequence: BB 257, forward primer in the first transmembrane domain, and BB 258, reverse primer in the seventh transmembrane domain. The single 780 bp fragment from both rat hippocampus and rat cerebellum were isolated from a 1% agarose gel, purified using a GENECLEAN III kit (BIO 101, Vista, CA) and sequenced using AmpliTaq DNA Polymerase, FS (Perkin Elmer). The sequence was run on an ABI PRISM 377 DNA Sequencer and analyzed using the Wisconsin Package (GCG, Genetics Computer Group, Madison, WI). This sequence was used to design PCR primers for the rat $GABA_BR2$ gene.

Construction and screening of a rat hypothalamic cDNA library

Poly A+ RNA was purified from rat hypothalamic RNA (Clontech) using a FastTrack kit (Invitrogen, Corp.). DS-cDNA was synthesized from 5 μg of poly A+ RNA according to Gubler and Hoffman (1983) with minor modifications. The resulting cDNA was ligated to BstXI adaptors (Invitrogen, Corp.) And the excess adapters removed by exclusion column chromatography. High molecular weight fractions of size-selected ds-cDNA were ligated in pEXJ.T7, an Okayama and Berg expression vector modified from pcEXV (Miller and Germain, 1986) to contain BstXI, other additional restriction sites, and a T7 promoter. A total of 100,000 independent clones with a mean insert size of 3.7 kb were

generated. The library was amplified on agar plates (Ampicillin selection) in 48 primary pools. Glycerol stocks of the primary pools screened for a rat $GABA_BR2$ gene by PCR using BB265, a forward primer from the loop between transmembrane domains 3 and 4 from the sequence determined above and BB266, a reverse primer from the sixth transmembrane domain from the sequence determined above. The conditions for PCR were 1 min at 94°C, 4 min at 68°C for 40 cycles, with a pre- and post-incubation of 5 min at 95°C and 7 min at 68°C, respectively. To determine which pools had the largest inserts, positive pools were screened by PCR using the vector primers BB172 or BB173, and a gene-specific primer BB265 or BB266. One positive primary pool, I-47, was subdivided into 24 pools of 1000 clones, and grown in LB medium overnight. Two μL of cultures were screened by PCR using primers BB172 and BB266. One positive subpool, I-47-4 was subdivided into 10 pools of 200 clones and plated on agar plates (ampicillin selection). Colonies were transferred to nitrocellulose membranes (Schleicher and Schuell, Keene, NH), denatured in 0.4 N NaOH, 1.5 M NaCl, renatured in 1M Tris, 1.5 M NaCl, and UV cross-linked. Filters were hybridized overnight at 40°C in a buffer containing 50 % formamide, 0.12 M Na_2HPO_4 (pH7.2), 0.25M NaCl, 7%SDS, 25 mg/L ssDNA and 10^6 cpm/mL of a cDNA probe corresponding to transmembrane domains 1 to 7 of rat $GABA_BR2$, labeled with [^{32}P]dCTP (3000Ci/mmol, NEN) using a random prime labeling kit (Boehringer Mannheim). Filters were washed 1x 5 min then 2x 20 min at room temperature in 2x SSC, 0.1%SDS then 3x 20 min at 50° in 0.1x SSC, 0.1% SDS and exposed to Biomax MS film (Kodak) for 3 hours. Four closely clustering colonies which appeared to hybridize were re-screened individually by PCR using primers BB265 and BB266, primers BB265 and BB55, primers BB265 and BB56, and primers BB266 and BB55. The conditions for PCR were 30

sec at 94°C, 2.5 min at 68°C for 32 cycles, with a pre- and post-incubation of 5 min at 95°C and 5 min at 68°C respectively. One positive colony, I-47-4-2, was amplified overnight in 10 mL TB media and processed for plasmid purification using a standard alkaline lysis miniprep procedure followed by a PEG precipitation. This plasmid was designated BO54 and partially sequenced using AmpliTaq DNA Polymerase, FS (Perkin Elmer). The sequence was run on an ABI PRISM 377 DNA Sequencer and analyzed using the Wisconsin Package (GCG, Genetics Computer Group, Madison, WI). BO54 was in the wrong orientation for expression in mammalian cells. To obtain a clone in the correct orientation, an *Eco*RI restriction fragment from BO54 was subcloned into the vector pEXJ. Transformants were screened by PCR using the primers BB56 and BB268 under the following conditions: 30 sec at 94°C, 2.5 min at 68°C for 32 cycles, with a pre- and post-incubation of 5 min at 95°C and 3 min at 68°C respectively. One transformant in the correct orientation was amplified overnight in 100 ml TB media and processed for plasmid purification using a standard alkaline lysis miniprep procedure followed by a PEG precipitation. This plasmid was designated BO55 and sequenced using AmpliTaq DNA Polymerase, FS (Perkin Elmer). Plasmid BO-55 was deposited with the ATCC on June 10, 1997, and was accorded ATCC Accession No. 209104. The sequence of BO-55 was determined using an ABI PRISM 377 DNA Sequencer and analyzed using the Wisconsin Package (GCG, Genetics Computer Group, Madison, WI).

Primers Used

BB257: 5'-CTCTCTGCCCTCACCATCCTCGGGAT-3' (Seq. ID No. 21)

BB258: 5'-GACTCCGGCTCGAATACCAGGCAGAG-3' (Seq. ID No. 22)

BB265: 5'-CCATGTTTGCAAAGACCTGGAGGGTCC-3' (Seq. ID No.

23)
BB266: 5'-GGTCACGCGTCAGGAAAGAGACAGCAG-3' (Seq. ID No. 24)
BB172: 5'-AAGCTTCTAGAGATCCCTCGACCTC-3' (Seq. ID No. 25)
BB173: 5'-AGGCGCAGAACTGGTAGGTATGGAA-3' (Seq. ID No. 26)
BB55: 5'-CTTCTAGGCCTGTACGGAAGTGTTA-3' (Seq. ID No. 27)
BB56: 5'-GTTGTGGTTTGTCCAAACTCATCAATG-3' (Seq. ID No. 28)
BB268: 5'-CTGCTGTCTCTTTCCTGACGCGTGACC-3' (Seq. ID No. 29).

Generation of DNA coding for rat $GABA_B$1b and $GABA_B$1a polypeptides

The gene encoding the rat $GABA_B$R1b polypeptide was obtained by screening the same rat hypothalamic library used for $GABA_B$R2 with primers based on the original publication of the clone by Kaupmann, et al., 1997. A partial clone lacking the first 55 nucleotides was identified and ligated to a PCR fragment containing the missing base pairs to obtain the full length clone. A restriction fragment containing the entire coding region of $GABA_B$R1b was subcloned into the mammalian expression vector pEXJ.T7 and designated "BO58". A rat $GABA_B$1a polypeptide clone was obtained by ligating a restriction fragment of the $GABA_B$1b clone, which contained the common region of the $GABA_B$1 gene, to a PCR product containing the $GABA_B$1a-specific 5' end.

In Situ Hybridization experiments for $GABA_B$R2 mRNA

Animals

Male Sprague-Dawley rats (Charles Rivers, Rochester, NY) were euthanized using CO_2, decapitated, and their brains immediately removed and rapidly frozen on crushed dry ice. Coronal sections of brain tissue were cut at 11 μm using a cryostat and thaw-mounted onto poly-L-lysine-coated slides and stored at -20°C until

use.

Tissue Preparation

Prior to hybridization, the tissues were fixed in 4% paraformaldehyde/PBS pH 7.4 followed by two washes in PBS (Specialty Media, Lavallette, NJ). Tissues were then treated in 5 mM dithiothreitol, rinsed in DEPC-treated PBS, acetylated in 0.1 M triethanolamine containing 0.25% acetic anhydride, rinsed twice in 2 x SSC, delipidated with chloroform then dehydrated through a series of graded alcohols. All reagents were purchased from Sigma (St. Louis, MO).

Radioactive *In Situ* Hybridization Histochemistry

Oligonucleotide probes, MJ79/80, corresponding to nucleotides 354-398 and MJ109/110, corresponding to nucleotides 952-991 of the rat $GABA_BR2$ cDNA, MJ94/95, corresponding to nucleotides 151-193 of the human $GABA_BR1a$ cDNA, and MJ83/84, corresponding to nucleotides 34-71 of the rat $GABA_BR1b$ cDNA were used to characterize the distribution of each polypeptides's respective mRNA. The oligonucleotides were synthesized using an Expedite Nucleic Acid Synthesis System (PerSeptive Biosystems, Framingham, MA) and purified using 12% polyacrylamide gel electrophoresis. Additionally, sense and antisense oligonucleotides corresponding to positions 1076-1120 of $GABA_BR1b$ (1424-1468 of $GABA_BR1a$) were used (BB403 and BB404).

The sequences of the oligonucleotides are:

For rat $GABA_BR2$:

Sense probe, MJ79:
5'- GCA ATA AAG TAT GGG CTG AAC CAT TTG ATG GTG TTT GGA GGC GT -3' (Seq. ID No. 36)

Antisense probe, MJ80:

5'- ACG CCT CCA AAC ACC ATC AAA TGG TTC AGC CCA TAC TTT ATT GC- 3' (Seq. ID No. 37)

Sense probe, MJ109:
5'- TTT GAG CCC CTG AGC TCC AAA CAA ATC AAG ACC ATC TCA G- 3' (Seq. ID No. 38)

Antisense probe, MJ110:
5'- CTG AGA TGG TCT TGA TTT GTT TGG AGC TCA GGG GCT CAA A- 3' (Seq. ID No. 39)

For human $GABA_BR1a$:

Sense probe, MJ94:
5'- AAG GCC ATC AAC TTC CTG CCT GTG GAC TAT GAG ATC GAA TAT G- 3' (Seq. ID No. 40)

Antisense probe, MJ95:
5'- CAT ATT CGA TCT CAT AGT CCA CAG GCA GGA AGT TGA TGG CCT T- 3' (Seq. ID No. 41)

For rat $GABA_BR1b$:

Sense probe, MJ83:
5'- TGG CCG CTG CCT CTT CTG CTG GTG ATG GCG GCT GGG GT - 3' (Seq. ID No. 42)

Antisense probe, MJ84:
5'- ACC CCA GCC GCC ATC ACC AGC AGA AGA GGC AGC GGC CA -3' (Seq. ID No. 43)

Sense probe, BB403:
5' - CCT TGG CTT TGG CCT TGA ACA AGA CGT CTG GAG GAG GTG GTC GTT -3' (Seq. ID No. 44)

Antisense probe, BB404:
5' - AAC GAC CAC CTC CTC CAG ACG TCT TGT TCA AGG CCA AAG CCA AGG -3'
(Seq. ID No. 45)

Probes were 3'-end labeled with [^{35}S]dATP (1200Ci/mmol, NEN, Boston, MA) to a specific activity of 10^9 dpm/μg using terminal deoxynucleotidyl transferase (Pharmacia, Piscataway, NJ). *In situ* hybridization was done with modification of the method described by Durkin, M, et al, 1995.

Nonradioactive *In Situ* Hybridization Histochemistry

Antisense/sense probes corresponding to nucleotides 354 - 398 of the rat $GABA_BR2$ cDNA, were 3'-end labeled with digoxigenin using TdT. The labeling reaction was carried out as outlined in the DIG/Genius System,(Boehringer Mannheim, Indianapolis, IN). Conditions used in ISHH with digoxigenin-labeled probes are the same as described above. The sections were rinsed in buffer 1, washing buffer (0.1 M Tris-HCl pH 7.5/0.15 M NaCl), pre-incubated in Blocking Solution (Buffer 1 , 0.1% Triton-X and 2% normal sheep serum) for 30 minutes and then incubated for 2 hours in Blocking Solution containing anti-digoxigenin-AP Fab fragment (Boehringer Mannheim) at 1:500 dilution followed by two 10 minute washes in Buffer 1. To develop color, sections were rinsed in Detection Buffer (0.1M Tris-HCl pH 9.5/0.15M NaCl/0.05 M $MgCl_2$) for 10 minutes and then incubated overnight in Detection Buffer containing 0.5 mM NBT, 0.1 mM BCIP, and 1 mM levamisole. After color development, slides were dipped in dH_2O and coverslipped using aqua mount.

Probe specificity was established by performing *in situ* hybridization on HEK293 cells transiently transfected with eukaryotic expression vectors containing the rat

$GABA_BR1b$ and human $GABA_BR1a$ DNA or no insert for transfection. Furthermore, two pairs of hybridization probes, sense and antisense, that were targeted to different segments of the $GABA_BR2$ mRNA were used for cells and rat tissues.

Quantification

The strength of the hybridization signal obtained in various region of the rat brain was graded as weak (+), moderate (++), heavy (+++) or intense (++++). These were qualitative evaluations for each of the polypeptide mRNA distributions based on the relative optical density on the autoradiographic film and on the relative number of silver grains observed over individual cells at the microscopic level.

Cell Culture

COS-7 cells are grown on 150 mm plates in DMEM with supplements (Dulbecco's Modified Eagle Medium with 10% bovine calf serum, 4 mM glutamine, 100 units/mL penicillin/100 μg/mL streptomycin) at 37°C, 5% CO_2. Stock plates of COS-7 cells are trypsinized and split 1:6 every 3-4 days.

Human embryonic kidney 293 cells are grown on 150 mm plates in DMEM with supplements (10% bovine calf serum, 4 mM glutamine, 100 units/mL penicillin/100μg/mL streptomycin) at 37°C, 5% CO_2. Stock plates of 293 cells are trypsinized and split 1:6 every 3-4 days.

Mouse fibroblast LM(tk-) cells are grown on 150 mm plates in D-MEM with supplements (Dulbecco's Modified Eagle Medium with 10% bovine calf serum, 4 mM glutamine, 100 units/mL penicillin/100 μg/mL streptomycin) at 37°C, 5% CO_2. Stock plates of LM(tk-) cells are trypsinized and split 1:10 every 3-4 days.

Chinese hamster ovary (CHO) cells are grown on 150 mm plates in HAM's F-12 medium with supplements (10% bovine calf serum, 4 mM L-glutamine and 100 units/mL penicillin/100 ug/mL streptomycin) at 37°C, 5% CO2. Stock plates of CHO cells are trypsinized and split 1:8 every 3-4 days.

Mouse embryonic fibroblast NIH-3T3 cells are grown on 150 mm plates in Dulbecco's Modified Eagle Medium (DMEM) with supplements (10% bovine calf serum, 4 mM glutamine, 100 units/mL penicillin/100 μg/mL streptomycin) at 37°C, 5% CO2. Stock plates of NIH-3T3 cells are trypsinized and split 1:15 every 3-4 days.

Sf9 and Sf21 cells are grown in monolayers on 150 mm tissue culture dishes in TMN-FH media supplemented with 10% fetal calf serum, at 27°C, no CO_2. High Five insect cells are grown on 150 mm tissue culture dishes in Ex-Cell 400™ medium supplemented with L-Glutamine, also at 27°C, no CO_2.

LM(tk-) cells stably transfected with the DNA encoding the polypeptides disclosed herein may be routinely converted from an adherent monolayer to a viable suspension. Adherent cells are harvested with trypsin at the point of confluence, resuspended in a minimal volume of complete DMEM for a cell count, and further diluted to a concentration of 10^6 cells/mL in suspension media (10% bovine calf serum, 10% 10X Medium 199 (Gibco), 9 mM $NaHCO_3$, 25 mM glucose, 2 mM L-glutamine, 100 units/mL penicillin/100 μg/mL streptomycin, and 0.05% methyl cellulose). Cell suspensions are maintained in a shaking incubator at 37°C, 5% CO_2 for 24 hours. Membranes harvested from cells grown in this manner may be stored as large, uniform batches in liquid nitrogen.

Alternatively, cells may be returned to adherent cell culture in complete DMEM by distribution into 96-well microtiter plates coated with poly-D-lysine (0.01 mg/mL) followed by incubation at 37°C, 5% CO_2 for 24 hours.

Generation of baculovirus

The coding region of DNA encoding the polypeptides disclosed herein may be subcloned into pBlueBacIII into existing restriction sites, or sites engineered into sequences 5′ and 3′ to the coding region of the polypeptides. To generate baculovirus, 0.5 μg of viral DNA (BaculoGold) and 3 μg of DNA construct encoding a polypeptide may be co-transfected into 2 x 10^6 *Spodoptera frugiperda* insect Sf9 cells by the calcium phosphate co-precipitation method, as outlined in by Pharmingen (in "Baculovirus Expression Vector System: Procedures and Methods Manual"). The cells then are incubated for 5 days at 27°C.

The supernatant of the co-transfection plate may be collected by centrifugation and the recombinant virus plaque purified. The procedure to infect cells with virus, to prepare stocks of virus and to titer the virus stocks are as described in Pharmingen's manual.

Transfection

All subtypes studied may be transiently transfected into COS-7 cells by the DEAE-dextran method, using 1 μg of DNA /10^6 cells (Cullen, 1987). In addition, Schneider 2 Drosophila cells may be cotransfected with vectors containing the gene, under control of a promoter which is active in insect cells, and a selectable resistance gene, eg., the G418 resistant neomycin gene, for expression of the polypeptides disclosed herein.

Stable Transfection

DNA encoding the polypeptides disclosed herein may be co-transfected with a G-418 resistant gene into the human embryonic kidney 293 cell line by a calcium phosphate transfection method (Cullen, 1987). Stably transfected cells are selected with G-418.

Radioligand binding assays

Transfected cells from culture flasks were scraped into 5 mL of Tris-HCl, 5mM EDTA, pH 7.5, and lysed by sonication. The cell lysates were centrifuged at 1000 rpm for 5 min. at 4°C, and the supernatant was centrifuged at 30,000 x g for 20 min. at 4°C. The pellet was suspended in binding buffer (50 mM Tris-HCl, 2.5 mM $CaCl_2$ at pH 7.5 supplemented with 0.1% BSA, 2μg/mL aprotinin, 0.5mg/mL leupeptin, and 10μg/mL phosphoramidon). Optimal membrane suspension dilutions, defined as the protein concentration required to bind less than 10% of the added labeled compound (typically a radiolabeled compound), were added to 96-well polypropylene microtiter plates containing labeled compound, unlabeled compounds (i.e., displacing ligand in an equilibrium competition binding assay) and binding buffer to a final volume of 250 μL. In equilibrium saturation binding assays membrane preparations were incubated in the presence of increasing concentrations of labeled compound. The binding affinities of the different compounds were determined in equilibrium competition binding assays, using labeled compound, such as 1 nM [^{3}H]-CGP54626, in the presence of ten to twelve different concentrations of the displacing ligand(s). Some examples of displacing ligands included GABA, baclofen, 3APMPA, phaclofen, CGP54626, and CGP55845. Mixtures of several unlabeled test compounds (up to about 10 compounds) may also be used in competition binding assays, to determine whether one of the mixture component

compounds binds to the polypeptide or receptor. Binding reaction mixtures were incubated for 1 hr at 30°C, and the reaction was stopped by filtration through GF/B filters treated with 0.5% polyethyleneimine, using a cell harvester. Where the labeled compound was a radiolabeled compound, the amount of bound compound was evaluated by gamma counting (for ^{125}I) or scintillation counting (for ^{3}H). Data were analyzed by a computerized non-linear regression program. Non-specific binding was defined as the amount of radioactivity remaining after incubation of membrane protein in the presence of excess unlabeled compound. Protein concentration may be measured by the Bradford method using Bio-Rad Reagent, with bovine serum albumin as a standard.

Cyclic AMP (cAMP) formation assay

The receptor-mediated inhibition of cyclic AMP (cAMP) formation may be assayed in transfected cells expressing the mammalian receptors described herein. Cells are plated in 96-well plates and incubated in Dulbecco's phosphate buffered saline (PBS) supplemented with 10 mM HEPES, 5mM theophylline, 2 μg/ml aprotinin, 0.5 mg/ml leupeptin, and 10 μg/ml phosphoramidon for 20 min at 37°C, in 5% CO_2. Test compounds are added and incubated for an additional 10 min at 37°C. The medium is then aspirated and the reaction stopped by the addition of 100 mM HCl. The plates are stored at 4°C for 15 min, and the cAMP content in the stopping solution measured by radioimmunoassay. Radioactivity may be quantified using a gamma counter equipped with data reduction software.

Generation of chimeric G-proteins

Chimeric G-proteins were constructed using standard mutagenesis methods(Conklin *et al.*, 1993). Two chimeras were constructed. The first comprises the

entire coding region of human Ga_q with the exception of the final 3' 15 nucleotides which encode the C-terminal 5 amino acids of Ga_{i3}. The second also comprises the entire coding region of human Ga_q with the exception of the final 3' 15 nucleotides which encode the C-terminal 5 amino acids of Ga_z. Sequences of both chimeric G-protein genes were verified by nucleotide sequencing. For the purposes of expression in oocytes, synthetic mRNA transcripts of each gene were synthesized using the T7 polymerase.

Phosphoinositide Assay

The agonist activities of GABA-B agonists were assayed by measuring their ability to generate phosphoinositide production in COS-7 cells transfected transiently with $GABA_BR1$, $GABA_BR2$, and chimeric $Ga_{q/z}$. Alternatively, COS-7 cells are transfected transiently with $GABA_BR1$, $GABA_BR2$, and other chimeric G-protein alpha subunits such as $Ga_{q/i2}$, $Ga_{q/i3}$, or $Ga_{q/o}$. Cells were plated in 96-well plates and grown to confluence. The day before the assay the growth medium was changed to 100 ml of medium containing 1% serum and 0.5 mCi [^{3}H]*myo*-inositol, and the plates were incubated overnight in a CO_2 incubator (5% CO_2 at 37°C).

Immediately before the assay, the medium was removed and replaced by 200 ml of PBS containing 10 mM LiCl, and the cells were equilibrated with the new medium for 20 min. The [^{3}H]inositol-phosphate (IP) accumulation was started by adding 22 ml of a solution containing the agonist. To the first two wells 22 ml of PBS were added to measure basal accumulation, and 10 different concentrations of agonist were assayed in the following 10 wells of each plate row. All assays were performed in duplicate by repeating the same additions in two consecutive rows. The plates were incubated in a CO_2 incubator for 30 min. The reaction was terminated by

removal of the buffer solution by blotting, followed by the addition of 100 μl of 50% (v/v) trichloroacetic acid (TCA), and 10 min incubation at 4°C.

The contents of the wells were then transferred to a Multiscreen HV filter plate (Millipore) containing Dowex AG1-X8 (200-400 mesh, formate form). The filter plates were prepared adding 100 ml of Dowex AG1-X8 suspension (50% v/v, water:resin) to each well. The filter plates were placed on a vacuum manifold to wash or elute the resin bed. Each well was washed 3 times with 200 μl of 5mM *myo*-inositol. The [^{3}H]-IPs were eluted into empty 96-well plates with 75 ml of 1.2 M ammonium formate/0.1 M formic acid. After the addition of 200 μl of scintillation cocktail (Optiphase Supermix; Wallac) to each well, [^{3}H]-Ips were quantified by counting on a Trilux 1450 Microbeta scintillation counter.

<u>Oocyte expression</u>

Female *Xenopus laevis* (Xenopus-1, Ann Arbor, MI) are anesthetized in 0.2% tricain (3-aminobenzoic acid ethyl ester, Sigma Chemical Corp.) and a portion of ovary is removed using aseptic technique (Quick and Lester, 1994). Oocytes are defolliculated using 3 mg/ml collagenase (Worthington Biochemical Corp., Freehold, NJ) in a solution containing 87.5 mM NaCl, 2 mM KCl, 2 mM $MgCl_2$ and 5 mM HEPES, pH 7.5. Oocytes are injected (Nanoject, Drummond Scientific, Broomall, PA) with 50-70 nl mRNA prepared as described below. After injection of mRNA, oocytes are incubated at 17 degrees for 3-8 days.

RNAs are prepared by transcription from: (1), linearized DNA plasmids containing the complete coding region of the gene, or (2), templates generated by PCR

incorporating a T7 promoter and a poly A^+ tail. From either source, DNA is transcribed into mRNA using the T7 polymerase ("Message Machine", Ambion).

The transcription template for the rat $GABA_BR1b$ gene was prepared by PCR amplification of the plasmid BO58 using the primers MJ23 and MJ47 (see below). The template for the rat $GABA_BR2$ gene was made by linearization of the plasmid BO56, rat $GABA_BR2$ insert from BO55 in the expression vector pEXJ.T7, with NotI.

Primers:

MJ23 5'
CCAAGCTTCTAATACGACTCACTATAGGGGAGACCATGGGCCCGGGGGG

ACCCTGTACC 3' (Seq. ID No. 30);

MJ47 5' $T_{(35)}$CACTTGTAAAGCAAATGTACTCGACTCC 3' (Seq. ID No. 31).

Genes encoding G-protein inwardly rectifying K^+ channels 1 and 4 (GIRK1 and GIRK4; "GIRKs") were obtained by PCR using the published sequences (Kubo et al., 1993; Dascal et al., 1993; Krapivinsky et al., 1995b) to derive appropriate 5' and 3' primers. Human heart cDNA was used as template together with the primers

5'-CGCGGATCCATTATGTCTGCACTCCGAAGGAAATTTG-3' (Seq. ID No. 32) and

5'-CGCGAATTCTTATGTGAAGCGATCAGAGTTCATTTTTC -3' (Seq. ID No. 33) for GIRK1 and

5'-GCGGGATCCGCTATGGCTGGTGATTCTAGGAATG-3' (Seq. ID No. 34) and

5'- CCGGAATTCCCCTCACACCGAGCCCCTGG-3' (Seq. ID No. 35) for GIRK4.

The BamH1 and EcoR1 restriction sites in each primer

pair were used to clone the PCR product into the expression vector pcDNA-Amp (Invitrogen). Plasmid vectors containing GIRK1 and GIRK4 are referred to as "JS1800" and "JS1741", respectively. The coding regions of both genes were sequenced and verified.

Oocyte electrophysiology

Dual electrode voltage clamp ("GeneClamp", Axon Instruments Inc., Foster City, CA) is performed using 3 M KCl-filled glass microelectrodes having resistances of 1-3 Mohms. Unless otherwise specified, oocytes are voltage clamped at a holding potential of -80 mV. During recordings, oocytes are bathed in continuously flowing (1-3 ml/min) medium containing 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM HEPES, pH 7.5 (ND96), or elevated K^+ containing 49 mM KCl, 49 mM NaCl, 1.8 mM $CaCl_2$, 2 mM $MgCl_2$, and 5 mM HEPES, pH 7.5 (hK). Drugs are applied either by local perfusion from a 10 μl glass capillary tube fixed at a distance of 0.5 mm from the oocyte, or for calculation of steady-state EC_{50}s, by switching from a series of gravity fed perfusion lines. Experiments are carried out at room temperature. All values are expressed as mean +/- standard error of the mean.

Concentration-response curves for agonists and antagonists were fitted with logistic equations of the form $I = 1/(1 + (EC_{50}/[Agonist])^n)$ for agonists and $I = 1/(1 + ([Antagonist]/IC_{50})^n)$ for antagonists, where I is current, where EC_{50} is the concentration of agonist that produced half-maximal activation, IC_{50} is the concentration of antagonist that produced half-maximal inhibition, and n the Hill coefficient. Fits were made with a Marquardt-Levenberg non-linear least-squares curve fitting algorithm.

Recording ion currents in mammalian cells

The ability of the rat $GABA_BR1$ and $GABA_BR2$ genes to activate GIRK currents in mammalian cells was investigated by transient transfection of HEK-293 cells followed by voltage clamp analysis of currents. HEK-293 cells were maintained in Dulbecco's modified Eagle medium (DMEM) plus 10% (v/v) bovine calf serum, 2% L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin and were incubated at 37° C in a humidified 5% CO_2 atmosphere. Cells were harvested twice each week by treatment with 0.25% trypsin/1 mM EDTA in Hank's Salts and re-seeded at 20% of their original density either into 75 cm^2 flasks (for passaging) or into 35 mm tissue culture dishes (for transfection and electrophysiology experiments).

HEK-293 cells, 40% - 80% confluent, were co-transfected with various combinations of 0.6 ug each of the following plasmids: pGreen Lantern-1 (Gibco/BRL, Gaithersburg, MD), human GIRK1 (JS1800), human GIRK4 (JS1741), rat $GABA_BR1b$ (BO58), and rat $GABA_BR2$ (BO55). Cells were transiently transfected using the Superfect Transfection Reagent from Qiagen (Valencia, CA) according to the manufacturer's instructions. Briefly, 3 μg total plasmid DNA were incubated with 22.5 μl Superfect Reagent in 100 μl serum-free DMEM for 5-10 minutes at room temperature. After addition of 600 μl complete DMEM, the DNA/Superfect mixture was transferred to cells growing in 35 mm dishes coated with poly-D-lysine and incubated for 2-4 hours at 37° C in a 5% CO_2 incubator. Subsequently, the dishes were washed once with phosphate-buffered saline and 2 ml complete DMEM was added. Cells were incubated for 24-72 hours at 37° C before performing electrophysiological measurements.

The whole-cell configuration of the patch-clamp technique was used with glass pipettes having resistances of 2-4 MΩ when filled with the pipette

solution. Solutions used were (in mM), $KMeSO_4$, 125; KCl, 5; NaCl, 5; $MgCl_2$, 2; EGTA, 11; HEPES, 10, pH 7.4; MgATP, 1.0; Na_2GTP, 0.2, for the pipette and NaCl, 130; KCl, 4; $CaCl_2$, 2; $MgCl_2$, 2; Glucose, 10; Sucrose, 10; HEPES, 10, pH 7.4 for the bath. GIRK currents were recorded in elevated K^+ solution containing 25 mM K^+ and a correspondingly lower concentration of Na^+. Voltage clamp recordings were made with an EPC-9 amplifier using Pulse+PulseFit software (HEKA Elektronik). Series resistances were kept below 10 Mohm and no attempt was made to provide series resistance compensation. Currents were low-pass filtered at 1 kHz and digitized at a rate of 5 kHz. Unless otherwise noted, experiments were performed at room temperature on cells voltage clamped at a holding potential of -70 mV. Application of agonists was realized using a gravity-fed, perfusion system consisting of six concentrically arranged microcapillary tubes (Jones et al. 1997). The time to complete solution exchange was about 100 ms. The bath was constantly perfused at a low rate with control solution.

All voltage clamp recordings were made from transfected cells visualized under epifluorescent lighting conditions utilizing a filter set designed for GFP (Zeiss Optics). Fluorescent cells were an excellent indication of transfection since they all exhibited some constitutive GIRK current activity in contrast to untransfected cells which displayed no measurable inward rectifier K^+ currents (data not shown).

Microphysiometry

$GABA_BR1$, $GABA_BR2$ or the combination, were transiently expressed in CHO-K1 cells by liposome mediated transfection according to the manufacturer's recommendations ("LipofectAMINE", GibcoBRL, Bethesda,

MD), and maintained in Ham's F-12 medium with 10% bovine serum. Cells were prepared for microphysiometric recording as previously described (Salon, J. A., et al., 1995). On the day of the experiment the cell capsules were transferred to the microphysiometer and allowed to equilibrate in recording media (low buffer RPMI 1640, no bicarbonate, no serum, Molecular Devices Corp.), during which a baseline was established. The recording paradigm consisted of a 100 ml/min flow rate and a 30 s flow interruption during which the rate measurement was taken. Challenges involved an 80 s drug exposure just prior to the first post-challenge rate measurement being taken, followed by two additional pump cycles. Acidification rates reported are expressed as a percentage increase of the peak response over the baseline rate observed just prior to challenge.

N-terminal deletion experiments

As a start to exploring the structural aspects of $GABA_BR2$ important for functional activity of the $GABA_BR1/R2$ receptor, N-terminal deletion experiments were performed on the $GABA_BR2$-HA construct (see below). All such deletion mutants caused a complete disruption of receptor activity as assessed by the measurement of GIRK currents in transfected HEK293 cells. In one such experiment, wildtype $GABA_BR2$-HA was digested with BglII restriction enzyme and religated. The BglII deletion mutant (M118) lacks 257 amino acids at the N-terminus, corresponding to positions 226-482. Using immunofluorescence, M118 was found to be expressed on the cell surface, similarly to the wildtype $GABA_BR2$-HA, yet when co-expressed with $GABA_BR1$ did not produce GIRK activation with 100 μM GABA. Thus, although we cannot yet identify specific amino acids contributing to receptor activity, it appears that the N-terminal

region comprising amino acids 226-482 is critically important either for dimer formation, ligand binding or conformational changes associated with signal transduction.

Construction of epitope-tagged polypeptides and confocal microscopy

Incorporation of sequences encoding the RGS6xHis or influenza virus hemagglutinin (HA) epitope into the $GABA_BR1$ and $GABA_BR2$ genes, respectively, was performed by PCR. Each epitope was positioned immediately before the stop codon in the appropriate gene. Both tagged genes were subcloned into pcDNA. Sequence analysis was used to confirm all PCR-derived portions of the construct. Forty-eight hours post-transfection HEK293 cells were fixed for 20 min in 4% paraformaldehyde in PBS, permeablized in PBS containing 2% BSA and 0.1% Triton X-100 and incubated with primary antibody for 1.5 h. Mouse monoclonal anti-RGS (Qiagen) and mouse anti-FLAG (Boehringer-Mannheim) were labeled with FITC-conjugated goat anti-mouse antibodies. Rat monoclonal anti-HA (Boehringer-Mannheim) was visualized with TRITC-conjugated rabbit anti-irat antibodies. Fluorescent images were obtained with a Zeiss LSM 410 confocal microscope using a 100x oil-immersion objective.

Immunoprecipitation and Western blotting

Forty-eight hours following transient transfection HEK293 cells were solubilized in lysis buffer containing (in mM): 50 Tris/Cl pH 7.4, 300 NaCl, 1.5 $MgCl_2$, 1 $CaCl_2$, protease inhibitors (Boehringer Mannheim tablets), 1% Triton X-100, and 10% glycerol. 1-2 mg of

protein was immunoprecipitated overnight at 4° C with either 0.5 μg rat monoclonal anti-HA antibody or 0.5 μg mouse monoclonal anti-4xHis antibody (Qiagen). Immune complexes were bound to 20 μl Protein-A agarose (Research Diagnostics, Inc.) for 2 h at RT. Protein-A pellets were washed twice with buffer containing Triton-X-100, then once without, and eluted with 80 μl Laemmli sample buffer containing 2% (w/v) SDS and 20 mM DTT. After heating for 3 min. at 70° C, 20 μl IP samples or 20 μg total protein was subjected to SDS-PAGE followed by Western blotting with either anti-HA or anti-4xHis antibody, followed by sheep anti-rat (Amersham) or goat anti-mouse (RDI) HRP-linked secondary antibodies, respectively. Proteins were visualized with enhanced chemiluminescent substrates (Pierce).

Alternatively, material for immunoprecipitations was obtained by sucrose gradient fractionation of the P1 pellet as described by Graham(Graham, 1984). To verify the enrichment of plasma membrane in the resulting "P1+" pellet, Na^+/K^+ ATPase in the P1+ and P2 (primarily microsomal and vesicular(Graham, 1984)) fractions was quantified by fluorescence detection of anti-alpha 1 subunit antibody (Research Diagnostics, Inc., clone 9A-5) on a phosphor imager (Molecular Dynamics). ATPase in P1+ fractions used for immunoprecipitations was found to be enriched >50 fold compared to P2 fractions.

Experimental Results

Novel GPCR sequences identified by BLAST search

The rat $GABA_BR1a$ amino acid sequence (Kaupmann *et al.* (1997) Nature 386:239) was used as a query to search the EST division of GenBank with BLAST. Two entries, T07621 and Z43654, had probability scores that suggested significant amino acid homology to the $GABA_BR1a$ polypeptide. T07621 had sequence homology from the beginning of the first transmembrane domain to the beginning of third transmembrane domain of the $GABA_BR1a$ polypeptide. Z43654 had sequence homology from the sixth transmembrane domain to the seventh transmembrane domain of the $GABA_BR1a$ polypeptide. The sequence documentation for T07621 and Z43654 was retrieved with Entrez (NCBI) and neither sequence was annotated as having homology to any 7-TM spanning protein.

These results were used to obtain a full-length human clone TL-266, comprising both of the sequences identified by the BLAST search. Sequence analysis of clone TL-266 revealed a complete coding region for a novel protein. A search of the GenEMBL database indicated that the most similar sequence was that of $GABA_BR1a$, followed by G protein-coupled receptors (GPCRs) of the metabotropic receptor superfamily. The nucleotide and deduced amino acid sequence of TL-267 are shown in Figures 1 and 2, respectively. The nucleotide sequence of the coding region is 57% identical to the rat $GABA_BR1a$ over a region of 1,686 bases. The longest open reading frame encodes an 898 amino acid protein with 38% amino acid identity to the rat $GABA_BR1a$ polypeptide. Hydropathy plots of the predicted amino acid sequence reveal seven hydrophobic regions that may represent transmembrane domains (TMs, data not shown), typical of the G protein-coupled

receptor superfamily. In the putative TM domains, $GABA_BR2$ exhibits 45% amino acid identity with the rat $GABA_BR1a$ polypeptide. The amino terminus of TL-266 has amino acid homology to the bacterial periplasmic binding protein, a common feature of the metabotropic receptors (O'Hara et al. (1993) Neuron 11:41-52).

Generation of rat $GABA_BR2$ PCR Product

Using PCR primers designed against the first and seventh transmembrane domains of the human $GABA_BR2$ sequence, BB257 and BB258, a 780 base pair fragment was amplified from rat hippocampus and rat cerebellum. Sequence from these bands displayed 90% nucleotide identity to the human $GABA_BR2$ gene. This level of homology is typical of a species homologue relationship in the GPCR superfamily.

Construction and Screening of a Rat Hypothalamic cDNA Library

To obtain a full-length rat $GABA_BR2$ clone, pools of a rat hypothalamic cDNA library were screened by PCR using primers BB265 and BB266. A 440 base pair fragment was amplified from 44 out of 47 pools. Vector-anchored PCR was performed to identify pools with the largest insert size. One positive primary pool, I-47, was subdivided into 24 pools of 1000 individual clones and screened by vector-anchored PCR. Seven positive subpools were identified and one, I-47-4, was subdivided into 10 pools of 200 clones, plated onto agar plates, and screened by southern analysis. Four closely clustering colonies that appeared positive were rescreened individually by vector-anchored PCR. One positive colony, I-47-4-2, designated BO54, was amplified as a single rat $GABA_BR2$ clone. Since vector-anchored PCR revealed that BO54 was in the wrong

orientation for expression, the insert was isolated by restriction digest and subcloned into the expression vector pEXJ. A transformant in the correct orientation was identified by vector-anchored PCR, and designated BO-55.

The nucleotide and deduced amino acid sequence of BO-55 are shown in Figures 3 and 4, respectively. BO-55 contains a 2.82 kB open reading frame and encodes a polypeptide of 940 amino acids. The nucleotide sequence of BO-55 is 89% identical to TL-267 in the coding region, with an overall amino acid identity of 98%. The proposed signal peptide cleavage site is between amino acids 40 and 41 (Nielsen et al., 1997).

A BLAST search of GenEMBL indicated that this sequence was most closely related to $GABA_BR1$, displaying 35% and 41% amino acid identities overall and within the predicted transmembrane domains, respectively (Fig. 10). The structural similarity to $GABA_BR1$ indicated that this sequence encodes a novel polypeptide, which we refer to as $GABA_BR2$. The next most related sequences were other members of the mGluR family, with 21-24% overall amino acid identities. Like $GABA_BR1$ and other members of the mGluR family (O'Hara, P. J., et al., 1998), $GABA_BR2$ contains a large N-terminal extracellular domain having regions of homology to bacterial periplasmic binding proteins.

<u>Distribution of $GABA_BR1$ or $GABA_BR2$ in cDNA libraries</u>

Three cDNA libraries were screened by PCR with primers directed to transmembrane regions of either $GABA_BR1$ or $GABA_BR2$. In a human hippocampal cDNA library both polypeptides were found in greater than 90% of the pools and in a rat hypothalamic cDNA library, again both polypeptides were found in greater than 90% of the

pools. In addition, within each of these two libraries, the respective frequency of $GABA_BR1$ and $GABA_BR2$ seems to be the same. However, in a rat spinal cord cDNA library, $GABA_BR1$ was found in 62.5% of the pools while $GABA_BR2$ was found in only 17.5% of the pools. Thus, while both polypeptide subtype appear to be present at high frequency in all three of the libraries, in the spinal cord library $GABA_BR2$ occurs at 3.6-fold lower frequency. These data point to the existence of an additional $GABA_B$-like peptide(s).

Results of Localization

Controls

The specificity of the hybridization of the $GABA_BR2$ probe was verified by performing *in situ* hybridization on transiently transfected HEK293 cells as described in Methods. The results indicate that hybridization to each of the individual $GABA_B$ polypeptides was specific only to the HEK293 cells transfected with each respective cDNA.

In addition, *in situ* hybridization on rat brain sections was performed using two hybridization probes targeted to different segments of the $GABA_BR2$ mRNA. In each case the pattern and intensity of labeling was identical in all regions of the rat CNS. Nonspecific hybridization signal was determined using the sense probes and was indistinguishable from background.

Localization of $GABA_BR2$ mRNA in rat CNS

The anatomical distribution of $GABA_BR2$ mRNA in the rat brain was determined by *in situ* hybridization. By light microscopy the silver grains were determined to be distributed over neuronal profiles. The results suggest that the mRNA for $GABA_BR2$ is widely distributed

throughout the rat CNS in addition to several sensory ganglia (Figures 19H-I). However, expression levels in the brain were not uniform with several regions exhibiting higher levels of expression such as the medial habenula, CA3 region of the hippocampus, piriform cortex, and cerebellar Purkinje cells (Figures 19A-F). Moderate expression levels were observed in the ventral pallidum, septum, thalamus, CA1 region of the hippocampus, and geniculate nuclei (Figures 19C,D,E). Lower expression of $GABA_BR2$ mRNA was seen in the hypothalamus, mesencephalon, and several brainstem nuclei (Figures 19D,F). GABAergic neurons and terminals are likewise widely distributed in the CNS (Mugnaini, E., et al., 1985). and the distribution of the $GABA_BR2$ mRNA correlates well with the distribution of GABAergic neurons. One exception is the substantia nigra which contains high densities of GABAergic neurons, yet very low expression of $GABA_BR2$ mRNA. Additionally, the anatomical distribution of $GABA_BR2$ mRNA is in concordance with previous reports of the distribution of $GABA_B$ binding sites obtained using [^{3}H]baclofen (Gehlert, D. R., et al., 1985), and [^{3}H]GABA (Bowery, N. J., et al., 1987). Furthermore, there was a high degree of similarity in the distribution and intensity of $GABA_BR2$ hybridization signal relative to those previously reported for $GABA_BR1$ (Bischoff, S., et al., 1997) (Figures 11, 12). Notable exceptions were the hypothalamus and caudate-putamen, where the expression of $GABA_BR2$ message appeared lower than that of $GABA_BR1$.

<u>Colocalization of $GABA_BR2$and $GABA_BR1b$ mRNAs in the rat CNS</u>

The results of the *in situ* hybridization studies using digoxygenin-labeled probe conjugated to alkaline phosphatase and the chromagen NBT/BCIP for the $GABA_BR2$

mRNA and an [^{35}S]dATP-labeled probe for the $GABA_BR1b$ mRNA indicated that coexpression of the $GABA_BR2$ mRNA and $GABA_BR1b$ mRNA did occur *in vivo* in neurons. In particular, colocalization was observed in cells of the medial habenula, hippocampus, and the cerebellar Purkinje cells. Likewise, there was evidence from the autoradiograms for potential overlapping distribution of the three known $GABA_B$ mRNAs in the olfactory bulb, throughout the entire neocortex, several hypothalamic nuclei, numerous thalamic nuclei and brain stem nuclei. However, the Purkinje cells of the cerebellum contained message for only $GABA_BR2$ and $GABA_BR1b$ and not the $GABA_BR1a$. Additionally, all three subtypes appear to be distributed throughout the gray matter of the spinal cord in all levels of the spinal cord.

The overlapping expression patterns of $GABA_BR1$ and $GABA_BR2$ transcripts in the brain suggested the possibility the polypeptides may be co-expressed in individual neurons and that both might be required for functional activity.

Oocyte expression

Postsynaptic inhibition of neurons by $GABA_B$ receptor activation is caused by the opening of inwardly rectifying K^+ channels (GIRK) (North, R. A., 1989; Andrade, R. et al., 1986; Gahwiler, B. H., et al., 1985; Luscher, C., et al., 1997). Oocytes expressing the combination of $GABA_BR1b$ and $GABA_BR2$ mRNAs together with GIRKs elicited large currents in response to 30 μM GABA (Table 1a and 1b). (Subsequent to the compilation of the data in Table 1a, experiments were done to make Table 1b.) GABA and baclofen evoked sustained currents of similar magnitude (Fig. 13B). In contrast, oocytes expressing transcripts encoding either $GABA_BR1a$, $GABA_BR1b$, or $GABA_BR2$ alone consistently failed to generate GIRK currents in response to high

concentrations of GABA (1 mM), baclofen (1 mM) or 3-APMPA (100 μM). Others have reported similar results with $GABA_BR1$ (Kaupmann, K. et al., 1997a; Kaupmann, K., et al., 1997b).

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Table 1a. Magnitude of GIRK currents stimulated by GABA in oocytes and HEK-293 cells expressing GIRK1 and GIRK4 and various combinations of rat $GABA_BR1$ and rat $GABA_BR2$.

	Oocytes mean (nA)	S.E.M.	(n)	HEK-293 mean (pA)	S.E.M.	(n*)
$GABA_BR1a$	0	0	(35)	-	-	-
$GABA_BR1b$	0	0	(15)	5	3	(3/26)
$GABA_BR2$	0	0	(19)	5	5	(1/6)
$GABA_BR1b$	1396	269	(7)	658	323	(9/10)
+ $GABA_BR2$						
$GABA_BR1b$	7	7	(2)	-	-	-
+ $GABA_BR2$						
+ PTX						

* number of cells responding / total number studied

Table 1b. Magnitude of GIRK currents stimulated by GABA in oocytes and HEK-293 cells expressing GIRK1 and GIRK4 and various combinations of rat $GABA_BR1$ and rat $GABA_BR2$.

	Oocytes mean (nA)	S.E.M.	(n)	HEK-293 mean (pA)	S.E.M.	(n*)
$GABA_BR1a$	0	0	(35)	-	-	-
$GABA_BR1b$	0	0	(23)	5	3	(5/26)
$GABA_BR2$	0.230	.13	(30)	.87	.87	(1/23)
$GABA_BR1b$	832	65	(65)	470	71	(70/81)
+ $GABA_BR2$						
$GABA_BR1b$	16	9	(3)	-	-	-
+ $GABA_BR2$						
+ PTX						

* number of cells responding / total number studied

Currents stimulated by GABA in oocytes injected with both $GABA_BR1b$ and $GABA_BR2$ mRNAs were completely blocked by the selective antagonist CGP55845 (1 μM) in a reversible fashion (data not shown). The potency of GABA and baclofen for eliciting GIRK currents was measured by performing steady-state cumulative concentration response assays on individual oocytes (Figure 6A). Like K^+ responses elicited by stimulation of native $GABA_B$ receptors (Lacy et al. 1988; Misgeld et al. 1995), responses in oocytes did not desensitize and could be faithfully reproduced by multiple agonist applications on single oocytes. Stimulation of inward current was concentration dependent for both GABA and baclofen. The EC_{50}s, 1.76 μM for GABA and 3.99 μM for baclofen (Figure 6B, Figure 7), agreed closely with those reported in the literature for native receptors (Lacy et al. 1988; Misgeld et al. 1995). Concentration-effect curves for GABA were shifted to the right, in an apparently competitive manner, by well characterized $GABA_B$-selective antagonists (Fig. 15B). Based on additional experiments, the EC_{50}'s are 1.32 μM for GABA and 3.31 μM for baclofen. The results to date are summarized in Table 2. Antagonist affinity estimates (Fig. 15B, Table 2) were similar to values reported in previous electrophysiological studies using brain tissue (Bon, C., et al., 1996; Seabrook, G. R., et al., 1990), as well as to those obtained by measuring displacement of radioligand from cells expressing $GABA_BR1$ alone (Kaupmann, K., et al., 1997a) (Table 2).

Table 2. Agonist and antagonist pharmacology in cells expressing $GABA_BR1$, $GABA_BR2$, or both.

Protein	Measurement	Agonist			Antagonist		
		GABA	Baclofen	3-APMPA	Phaclofen	CGP54626	CGP55845
$GABA_BR1$+$GABA_BR2$	pEC_{50}[1], pK_B[2]	5.88 ±0.01	5.48 ±0.05	7.29 ±0.02	3.80 ± 0.03[4]	7.48 ±0.05	8.60 ±0.09
$GABA_BR1$	pK_i[3]	4.6	4.3	5.2	>3.0	8.95	8.7

[1] n = 6-8 oocytes except for GABA; n = 20 oocytes.

[2] Measured using GABA as agonist; n = 4-6 oocytes.

[3] Displacement of [^{3}H]-CGP54626 from COS-7 cells expressing $GABA_BR1$; n = 3.4

[4] IC_{50} using EC_{50} concentration of GABA.

Evidence that GABA-induced currents were mediated by GIRK channels included: 1) dependency on elevated external K^+, 2) strong inward rectification of the current-voltage (I/V) relation, 3) reversal potential (-23.3 mV) close to the predicted equilibrium potential for K^+ (-23 mV), and 4) sensitivity to block by 100 μM Ba^{++} (Figure 8).

Three oocytes were injected with pertussis toxin (2 ng/oocyte) 6 h before voltage clamping. GABA-stimulated currents were abolished in these oocytes (Table 1a and 1b), suggesting that receptor activation of GIRKs was mediated by G-proteins G_i or G_o. Analogous results have been obtained by others expressing D2 dopamine receptors with GIRKs in oocytes (Werner et al. 1996).

GABA responses in co-transfected HEK-293 cells

To verify that both gene products, $GABA_BR1b$ and $GABA_BR2$, are also required for expression of functional $GABA_B$ receptors in mammalian cells, voltage clamp recordings were obtained from HEK-293 cells transiently transfected with various combinations of each gene along with GIRKs. Cells transfected with a combination of $GABA_BR1b$ (BO58) and $GABA_BR2$ (BO55) plus GIRKs consistently produced large K^+ currents in response to 100 μM GABA (9 of 10 cells tested, Table 1a and 70 of 81 cells tested, Table 1b). Large amplitude currents were also observed when $GABA_BR2$ was paired with the $GABA_BR1a$ splice variant (1046 " 247 pA; n = 9). In contrast, cells transfected with only one of the $GABA_B$ genes plus GIRKs responded either not at all or only very weakly to GABA (Table 1a and 1b). Small agonist-evoked currents (10-50 pA) were observed in 5 of 26 cells expressing $GABA_BR1$; similar weak currents were evoked in 1 of 23 cells expressing $GABA_BR2$.

GABA-elicited currents in doubly transfected cells were completely blocked by 100 μM Ba^{++} or the competitive antagonist CGP55845 at 1 μM (Figure 9). The EC_{50} for GABA stimulation of GIRKs in HEK-293 cells was determined using similar methods as for oocytes. The EC_{50}, 3.42 μM, was comparable to that measured in oocytes (1.76 μM; further experiments gave 1.32 μM). Thus, whether in *Xenopus* oocytes or HEK-293 cells, the behavior of the $GABA_B$ receptor is the same. Co-expression of both $GABA_B$R1b and $GABA_B$R2 is required to observe activation of the receptor by GABA.

To determine if co-expressed $GABA_B$R1/R2 could mediate a cellular response in the absence of exogenously supplied GIRKs, we transiently co-transfected CHO cells with $GABA_B$R1 and $GABA_B$R2 and measured agonist-evoked extracellular acidification using a microphysiometer. Baclofen stimulated a 9-fold increase in acidification rate (Fig 16) which was blocked by 100 nM CGP55845 and by pretreatment with PTX (not shown). This response was absent in cells expressing either protein alone. Since GIRK activity is undetectable in wild-type CHO cells (Krapivinsky, G., et al., 1995b) we conclude that GIRK expression is not a prerequisite for signal generation by $GABA_B$R1/R2.

$GABA_B$R1/$GABA_B$R2 signaling through chimeric G-proteins

Chimeric G-proteins have been used to "switch" the coupling pathway of a GPCR from one that normally inhibits adenylyl cyclase to one that activates phospholipase C (Conklin *et al.*, 1993). With the aim of developing an assay based on Ca^{++} or some other signal amenable to high throughput screening, we employed a $Ga_{q/i3}$ chimera to obtain Ca^{++}-induced Cl^- responses in oocytes. Oocytes were injected with $GABA_B$R1 and $GABA_B$R2 mRNAs as previously described. 2-3 days later oocytes were injected again with 50 pg of

$Ga_{q/i3}$ mRNA and recorded under voltage clamp conditions. In response to GABA (0.1 - 1 mM) 88% of these oocytes produced rapidly desensitizing inward currents (454 ± 92 nA; n = 14) typical of those stimulated by receptors that normally couple to Ga_q. In contrast, oocytes injected with only the $GABA_BR1/GABA_BR2$ combination (n > 100), or $GABA_BR1$ plus $Ga_{q/i3}$ (n = 4) failed to produce currents.

$GABA_B$ agonists also resulted in concentration-dependent stimulation of phosphoinositide production in COS-7 cells transfected transiently with $GABA_BR1$, $GABA_BR2$, and the chimeric G-protein $Ga_{q/z}$. The concentration of agonist evoking 50% of its maximum response (EC_{50}) and fold stimulation over basal were: GABA (EC_{50} = 1.8 μM; 2.4 fold); baclofen (1.7 μM; 1.8 fold); 3-aminopropylmethylphosphinic acid (EC_{50} = 0.11 μM; 2.2 fold). These results indicate that G-protein chimeras, in particular $Ga_{q/z}$ and $Ga_{q/i3}$, are useful for directing $GABA_B$ receptor stimulation to a phosphoinositide- or Ca^{++}-based assay.

A comparison of the pharmacological properties of $GABA_BR1$ and $GABA_BR2$ using radioligand binding revealed that membranes from HEK293 or COS-7 cells expressing $GABA_BR1$, but not those expressing $GABA_BR2$, were labeled by the high affinity antagonist [^{3}H]-CGP54626[21] (Table 2), indicating that the polypeptides are pharmacologically distinct. Neither was labeled by the agonists [^{3}H]-GABA or [^{3}H]-baclofen. Furthermore, with the available ligands (GABA, baclofen, APMPA, phaclofen, CGP54626, CGP-55845 and SCH-50911) the binding profile of membranes from cells co-transfected with $GABA_BR1/R2$ was not different from those transfected with $GABA_BR1$ alone. The absence of detectable high affinity agonist binding to $GABA_BR1/R2$, as well as to $GABA_BR1b$, constitutes a notable distinction from the $GABA_B$ binding profile in the CNS and may reflect the

absence of an essential, as yet undefined G-protein or accessory protein.

The molecular mechanism by which protein co-expression confers functional activity is unknown. We noted that varying the ratios of $GABA_BR1/R2$ cDNAs from 1/100 to 100/1 in HEK293 cells resulted in a symmetrical fall off in response amplitude (Fig.14B). This suggests that a 1:1 protein stoichiometry may be critical, and caused us to postulate that the polypeptides are forming a heteromeric association. Biochemical evidence supports the idea that certain GPCRs can exist as homodimers (Hebert, T.E., et al., 1996; Cvejic, S., et al., 1997; Ciruela, F., et al., 1995; Avissar, S., et al., 1983; Romano, C., et al., 1996), but the functional significance of this has been largely unexplored (Hebert, T.E., et al., 1996; Wreggett, K.A., et al., 1995). The possibility of a physical association was investigated using epitope-tagged versions of $GABA_BR1$ (RGS6xH tag) and $GABA_BR2$ (HA tag). C-terminal modification did not appear to alter the function of either polypeptide; maximal current amplitudes (Fig. 14B) and EC_{50} values for GABA (4.97 μM, n = 5) were unchanged compared to HEK293 cells expressing the wild-type $GABA_BR1/R2$ receptor combination (3.42 μM, n = 5). The subcellular distribution of epitope-tagged proteins was examined in transfected cells by fluorescence microscopy. When expressed individually, $GABA_BR1^{RGS6xH}$ and $GABA_BR2^{HA}$ were localized throughout the plasma membrane. Optical sectioning of antibody-labeled cells by confocal microscopy confirmed the membrane localization pattern, with less labeling in the cytoplasm and none in the nucleus. In co-transfected cells there was a striking overlap in the distribution of the two epitope tags (Fig. 17A-17C). Both proteins were prominently expressed on the plasma

membrane. Furthermore, co-localization occurred within the cytoplasm, suggesting that $GABA_BR1$ and $GABA_BR2$ assemble in the endoplasmic reticulum. In contrast, the cellular distribution of an unrelated GPCR, NPY Y5, differed considerably from that of $GABA_BR2$ (Fig. 17D), suggesting specificity in the association of $GABA_BR2$ with $GABA_BR1$.

Western blots of whole cell extracts from cells expressing $GABA_BR1^{RGS6xH}$, $GABA_BR2^{HA}$ or both, exhibited bands close to the predicted molecular weights of the two proteins (92 kD for $GABA_BR1$, 97 kD for $GABA_BR2$) and additional bands corresponding to the predicted molecular weights of receptor dimers (Fig. 18A,B). To determine if $GABA_BR1$ and $GABA_BR2$ co-associate in a heteromeric complex, we immunoprecipitated solubilized material from cells expressing both polypeptides. $GABA_BR2^{HA}$ was detected in material immunoprecipitated using either anti-His or anti-HA antibodies (Fig. 18). To determine if $GABA_BR1b$ and $GABA_BR2$ co-associate in a heteromeric complex, we performed immunoprecipitations using membrane fractions enriched in plasma membrane as determined by the presence of Na^+/K^+ ATPase (Figure 20A). In co-transfected cells only, $GABA_BR2^{HA}$ was detected in material immunoprecipitated using antibodies specific for the $GABA_BR1^{RGS6xH}$ protein (Figure 20B). This result confirms that both $GABA_BR1$ and $GABA_BR2$ are correctly targeted to the plasma membrane of HEK293 cells, and that the two proteins exist in a heteromeric complex, perhaps as heterodimers, on the membrane surface.

Experimental Discussion

A gene has been cloned that shows 38% overall identity at the amino acid level with the recently cloned $GABA_BR1$ polypeptide. Important predicted features of the new gene product include 7 transmembrane spanning regions, and a large extracellular N-terminal domain. Like the $GABA_BR1$ gene product, $GABA_BR2$ by itself does not promote the activation of cellular effectors such as GIRKs. When co-expressed together, however, the two permit a $GABA_B$ receptor phenotype that is quite similar to that found in the brain. The functional attributes of this reconstituted receptor include: 1) robust stimulation of a physiological effector (GIRKs), 2) EC_{50}s for GABA and baclofen in the same range as for $GABA_B$ receptors previously studied in the CNS, 3) antagonism by the high affinity selective antagonist CGP55845, and 4) inhibition of receptor function by pertussis toxin. These attributes are not observed when either $GABA_BR1$ or $GABA_BR2$ is expressed alone.

Our data indicate that $GABA_BR1$ and $GABA_BR2$ associate as subunits to produce a single pharmacologically and functionally defined receptor. Consistent with this view, double labeling *in situ* hybridization experiments provided evidence that $GABA_BR1$ and $GABA_BR2$ mRNAs are co-expressed in individual neurons and populations of neurons in several regions of the nervous system including hippocampal pyramidal cells (Fig. 21), cerebellar Purkinje cells (Fig. 12A,B) and sensory neurons in mesencephalic trigeminal nucleus (Fig. 21) and dorsal root ganglia. This co-localization pattern of $GABA_BR1$ and R2 transcripts predicts that $GABA_B$ receptors on these cells are comprised of $GABA_BR1/R2$ heteromers. Other as yet unidentified $GABA_B$ receptor homologues may associate elsewhere to produce novel

subtypes. For example, the low level of expression of $GABA_BR2$ mRNA relative to $GABA_BR1$ in caudate putamen and hypothalamus (Fig. 11A,B) raises the possibility that other $GABA_B$ receptor homologues may associate with $GABA_BR1$ to produce novel subtypes in these regions. Conclusive evidence that functional $GABA_B$ receptors exist in vivo as multimers will await immunofluorescence studies with specific antibodies.

The recent cloning of a family of accessory proteins that modify the binding and functional properties of a calcitonin-receptor-like receptor (McLarchie, et al., 1998) demonstrates that some 7-TM spanning proteins require additional unrelated proteins to reconstitute native GPCR activity. $GABA_BR1$ and $GABA_BR2$ are the first examples of 7-TM proteins for which activity is dependent on an interaction with another member within the same family of proteins. There will be considerable interest in whether other GPCRs are formed by heteromeric complexes of related 7-TM proteins. Many members of the superfamily of GPCRs, such as D_3, $5\text{-}HT_5$, and olfactory receptors, do not function well in heterologous expression systems and may require related partners to generate native receptor function (Nimschinsky, et al., 1997). The growing list of receptors that have been reported to exist as homodimers (Ciruela, F., et al., 1995; Cvejic, S., et al., 1997; Hebert, T.E., et al, 1996; Romano, C., et al., 1996; Maggio, R., et al., 1996) points to the likelihood that both homomeric and heteromeric assemblies are more widespread among GPCRs than previously thought.

There are several possible explanations for why two genes are required for full function of the $GABA_B$ receptor. One possible explanation is that the two gene products function together as a heterodimer having

high affinity agonist and antagonist binding sites. Currently, there is no precedent for heterodimerization of GPCRs. There is evidence that certain GPCRs, for example the mGluR5 receptor, can form homodimers via cystine disulfide bridges in the N-terminal domain (Romano et al., 1996). Significantly, synthetic peptides that inhibit homodimerization of beta2-adrenergic receptors also reduce agonist stimulation of adenylyl cyclase activity (Hebert et al., 1996). Useful parallels may be drawn from other classes of receptors where heterodimeric structures are well-known. For example, the NMDA (glutamate) receptor is comprised of two principal subunits, neither of which alone permits all of the native features of the receptor (see Wisden and Seeburg, 1993). $GABA_B$ receptors may be comprised similarly of two (or more) peptide subunits, such as $GABA_BR1$ and $GABA_BR2$, that form a quaternary structure having appropriate binding sites for agonist and G-protein.

A role for $GABA_BR2$ in modulating sensory information is suggested by *in situ* hybridization histochemistry which revealed the expression of $GABA_BR2$ mRNA in relay nuclei of several sensory pathways. In the olfactory and visual pathways $GABA_BR2$ appears to be in a position to modulate excitatory glutamatergic projections from the olfactory bulb and retina $GABA_BR2$ mRNA was observed in the target regions of projection fibers from the main olfactory bulb, including the olfactory tubercle, piriform and entorhinal cortices and from the retina, for instance the superior colliculus (Figures 19A,B; Table 3).

The ability to modulate nociceptive information might be indicated not only by the presence of $GABA_BR2$ transcripts in somatic sensory neurons of the

trigeminal and dorsal root ganglia (Figures 19H-I) but also by being present in the target regions of nociceptive primary afferent fibers, including the superficial layers of the spinal trigeminal nucleus and dorsal horn of the spinal cord (Figures 19F-G). Again, in each of these loci $GABA_BR2$ has been shown to be in a position to potentially modulate the influence of excitatory glutamatergic nociceptive primary afferents. In both ganglia, microscopic examination indicated that the hybridization signal did not appear to be restricted to any one size cell and was distributed evenly over small, medium and large ganglion cells. Thus, $GABA_BR2$ may be able to influence various sensory modalities. Expression levels appeared to be higher in the ganglion cells of the dorsal root with light to moderate expression in the trigeminal ganglia.

$GABA_BR2$ mRNA was likewise observed to be expressed in the vestibular nuclei which are target regions of inhibitory GABAergic Purkinje cells and also in the Purkinje cells themselves, suggesting that $GABA_BR2$ may be important in the mediation of planned movements (Figure 19F).

Moderate expression of $GABA_BR2$ transcripts throughout the telencephalon indicate a potential modulatory role in the processing of somatosensory and limbic system (entorhinal cortex) information, in addition to modulating visual (parietal cortex) and auditory stimuli (temporal cortex) as well as cognition. Furthermore, modulation of patterns of integrated behaviors, such as defense, ingestion, aggression, reproduction and learning could also be attributed to this receptor owing to its expression in the amygdala (Table 3). The high levels of expression in the thalamus suggest a possible regulatory role in the

transmission of somatosensory (nociceptive) information to the cortex and the exchange of information between the forebrain and midbrain limbic system (habenula). The presence of $GABA_BR2$ mRNA in the hypothalamus indicates a likely modulatory role in food intake, reproduction, the expression of emotion and possibly neuroendocrine regulation (Figure 19D). A role in the mediation of memory acquisition and learning may be suggested by the presence of the $GABA_BR2$ transcript throughout all regions of the hippocampus and the entorhinal cortex (Figure 19D).

Table 3. Distribution of rGABA$_B$R2, rGABA$_B$R1a, and GABA$_B$1b mRNA in the rat CNS. The strength of the hybridization signal for each of the respective mRNAs obtained in various regions of the rat brain was graded as weak (+), moderate (++),heavy (+++) or intense (++++)and is relative to the individual polypeptides.

Region	GABA$_B$R2	GABA$_B$R1a*	GABA$_B$R1b*	Potential Application
Olfactory bulb				Modulation of olfactory sensation
internal granule layer	+	++	++	
glomerular layer	+	++	++	
external plexiform layer	-	-	-	
mitral cell layer	-	+	++	
anterior olfactory n	++	++	++	
olfactory tubercle	+	++	+++	
Islands of Calleja	-	++	+++	
Telencepha-lon				Sensory integration
taenia tecta	++	++	++	
frontal cortex	++	++	++	
orbital cortex	++	++	++	
agranular insular cortex	+++	++	++	

Region	$GABA_BR2$	$GABA_BR1a$*	$GABA_BR1b$*	Potential Application
cingulate cortex	++	++	+	
retrosple-nial cortex	++	++	+	
parietal cortex	++	++	++	Processing of visual stimuli
occipital cortex	++	++	++	
temporal cortex	++	++	++	Processing of auditory stimuli
perirhinal cortex	++	++		
entorhinal cortex	++	++	++	Processing of visceral information
dorsal endopiriform n	++	++	++	
piriform cortex	+++	+++	+++	Integration/ transmission of incoming olfactory information
Basal Ganglia				
accumbens n	+	++	++	Modulation of dopaminergic function
caudate-putamen	+	+	++	Sensory/motor integration
globus pallidus	+	-	+	

Septum				
Region	**$GABA_BR2$**	**$GABA_BR1a$***	**$GABA_BR1b$***	**Potential Application**
medial septum	++	++	+	Cognitive enhancement via cholinergic system
lateral septum	++	+	++	Modulation of integration of stimuli associated with adaptation
septohippo-campal n	+	+	+++	
diagonal band n	++	++	++	
ventral pallidum	++	+	+	
Amygdala				Anxiolytic (activation - reduction in panic attacks) appetite, depression
basolateral n	++	+	+	
medial amygdaloid n	+	+	+	Olfactory amygdala
basomedial n	+	+		
central n	+++	-	+	
anterior cortical n	+	+	+	
postero-medial cortical n	++	+	+	
bed n stria terminalis	++	+	++	

Region	$GABA_BR2$	$GABA_BR1a$*	$GABA_BR1b$*	Potential Application
zona incerta	+	+	+	
Hippocampus				Memory consolidation and retention
CA1, Ammon's horn	++	+++	+++	
CA2, Ammon's horn	++++	+++	+++	
CA3, Ammon's horn	++++	+++	+++	Facilitation of LTP
subiculum	+	+++	+++	
parasubiculum	++	++	++	
presubiculum	++	++	++	
dentate gyrus	++++	+++	++	
polymorph dentate gyrus	+++	+++	++	
Hypothalamus				
suprachiasmatic n	+	++	ND	
median preoptic area	+	+	++	Regulation of gonadotropin secretion and reproductive behaviors
paraventricular n	+	++	++	Appetite/obesity
arcuate n	++	++	++	
anterior hypoth, post	+	+		
lateral hypoth	+	+	++	
ventromedial n	+	++	+++	
periventricular n	+	+	+	
supraoptic n	+	++	+	Synthesis of OXY and AVP

Region	$GABA_BR2$	$GABA_BR1a$*	$GABA_BR1b$*	Potential Application
supramammillary n	++	++	++	Modulation of hypothalamic projections to cortex
premammillary n	+	+	+	
medial mammillary n	+	++	+	
Thalamus				Analgesia/Modulation of sensory information
paraventricular n	++	+	++	Modulation of motor and behavioral responses to pain
centromedial n	++	+	++	Modulation of motor and behavioral responses to pain
paracentral n.	++	+	++	
parafascicular n	++	+	++	Modulation of motor and behavioral responses to pain
anterodorsal n	+++	+	++	Modulation of eye movement
laterodorsal n	+++	+	++	
lateral posterior n	++	+	++	

Region	$GABA_BR2$	$GABA_BR1a$*	$GABA_BR1b$*	Potential Application
reuniens n	+++	+	++	Modulation of thalamic input to ventral hippocampus and entorhinal ctx
rhomboid n	+++	+	++	
medial habenula	++++	+	++++	Anxiety/sleep disorders/ analgesia in chronic pain
lateral habenula	+	+	+++	
ventrolateral n	+++	+	++	
ventromedial n	+++	++	++	
ventral posterolateral n	+++	+	++	
reticular n	++	+	+	Alertness /sedation
lateral geniculate n	++	+	++	Modulation of visual perception
medial geniculate	++	+	++	Modulation of auditory system
subthalamic n	++	++	++	
Mesencephalon				
superior colliculus	+	+	+	Modulation of vision
inferior colliculus	+	+	+	
central gray	+	+	+	Analgesia
dorsal raphe	+	++	+	

Region	$GABA_BR2$	$GABA_BR1a$*	$GABA_BR1b$*	Potential Application
deep mesencephalic n	+	+	+	
oculomotor n	+			
pontine n	+++		++	
retrorubral field	+			
ventral tegmental area	+	++	++	Modulation of the integration of motor behavior and adaptive responses
substantia nigra, reticular	+	+	+	Motor control
substantia nigra, compact	++	++	++	
interpeduncular n	++	ND	ND	Analgesia
Myelencephalon				Analgesia
raphe magnus	++		++	
raphe pallidus	+	++	ND	
principal trigeminal	+	+		
spinal trigeminal n	+	+	+	
pontine reticular n	++	+	++	
parvicellular reticular n	+	++	++	

Region	GABA$_B$R2	GABA$_B$R1a*	GABA$_B$R1b*	Potential Application
locus coeruleus	++	++	++	Modulation of NA transmission
parabrachial n	+	++	+	Modulation of visceral sensory information
vestibular n	+	++	+	Maintenance of balance and equilibrium
gigantocell-ular reticular n	+	++	++	Inhibition and disinhibition of brainstem
prepositus hypoglossal n	+	+++	++	Position and movement of the eyes/ Modulation of arterial pressure and heart rate
ventral cochlear n	++	+	ND	
n soltary tract	++			Hypertension
A5 Nor-adrenaline cells	+	ND	ND	
facial n(7)	+	++	+	
Cerebellum				Motor coordina-tion, Autism
granule cell layer	+	+	+	
Purkinje cells	++	-	++	

Region	$GABA_BR2$	$GABA_BR1a$*	$GABA_BR1b$*	Potential Application
Spinal cord				Analgesia
dorsal horn	+	++	+	
ventral horn	+	++	+	
trigeminal ganglion	++	+++	+	Nociception
dorsal root ganglion	++++	+++	ND	Nociception

ND = not determined

*Bischoff S *et al.*

List of Abbreviations

7	facial n
ac	anterior commisure
Acb	accumbens n
ACo	anterior cortical amygdaloid n
AI	agranular insular cortex
AON	anterior olfactory n
APir	amygdalopiriform transition area
APT	anterior pretectal n
Arc	arcuate hypothalamic n
BLA	basolateral amygdaloid n
CA1-3	Fields of Ammon's horn
cc	corpus callosum
Cg	cingulate cortex
CeA	central amygdaloid n
CPu	caudate-putamen
DG	dentate gyrus
DLG	dorsal lateral geniculate n
DpMe	deep mesencephalic n
Ent	entorhinal cortex
Gi	gigantocellular reticular n
Gr	granule cll layer, cerebellum
GrO	granule layer olf. bulb
FrA	frontal association cortex
GP	globus pallidus
HDB	horizontal diagonal band
LA	lateral amygdaloid n
LH	lateral hypothalamus
LO	lateral orbital cortex
LV	lateral ventricle
M1	primary motor cortex
MeAD	medial amygdaloid n, anterodorsal
MG	medial geniculate

MHb	medial habenular n
MPO	medial preoptic n
PC	Purkinje cell layer of the cerebellum
PF	parafascicular n
Pir	piriform cortex
PMCo	posteromedial cortical amygdaloid n
Pr	prepositus n
PVA	paraventricular thalamic n
RS	retrosplenial cortex
S	subiculum
SFi	septofimbrial n
SI	substantia innominata
SNc	substantia nigra, compact
STh	subthalamic n
Sp5	spinal trigeminal n
TT	tenia tecta
Ve	vestibular n
VTA	ventral tegmental area

Potential therapeutic application for $GABA_B$ agonists and antagonists

Agonists

Antinociception

A potential $GABA_B$ agonist application may in antinociception. The inhibitory effects of GABA and $GABA_B$ agonists are thought to be predominantly a presynaptic mechanism on excitation-induced impulses in high threshold Ad and C fibers on primary afferents. This effect can be blocked by $GABA_B$ antagonists (Hao, J-H., et al., 1994). Baclofen's spinal cord analgesic effects have been well documented in the rat, though it has not been as effective in human. However, baclofen

has been successful in the treatment of trigeminal neuralgia in human.

The localization of the $GABA_BR2$ mRNA in the superficial layers of the spinal cord dorsal horn, the termination site for primary afferents, as well as their cells of origin in the dorsal root and trigeminal ganglia position the $GABA_BR1/R2$ receptor appropriately for mediating the agonist effects.

Drug Addiction

It has been suggested that GABA agonists may have some potential in the treatment of cocaine addiction. A role for the action of psychostimulants in the mesoaccumbens dopamine system is well established. The ventral pallidum receives a GABAergic projection from the nucleus accumbens and both regions contain $GABA_BR2$ transcripts. GABA receptors were shown to have an inhibitory effect on dopamine release in the ventral pallidum. Phaclofen acting at these receptors resulted in increased dopamine release and baclofen was shown to attenuate the reinforcing effects of cocaine. (Roberts, D. C. S., et al.,1996; Morgan,A.E. et al.)

Micturition

There is a potential application for $GABA_B$ agonists in the treatment of bladder dysfunction. Baclofen has been used in the treatment of detrussor hyperreflexia through inhibition of contractile responses. In addition to a peripheral site of action for $GABA_B$ agonists, there is also the possibility for a central site. The pontine micturition center in the brainstem is involved in mediating the spinal reflex pathway, via Onuf's nucleus in the sacral spinal cord. Support for

possible application of $GABA_B$ agonists in the treatment of bladder dysfunction may be augmented by presence of $GABA_BR2$ mRNA in the various nuclei involved in the control of the lower urinary tract function.

Antagonists

Memory Enhancement - Alzheimer's Disease

$GABA_B$ antagonists may have a potential application in the treatment of Alzheimer's Disease. The blockade of $GABA_B$ receptors might lead to signal amplification and improvement in cognitive functions resulting from an increased excitability of cortical neurons via amplification of the acetycholine signal. Additionally, memory may be enhanced by $GABA_B$ antagonists which have been shown to suppress late IPSPs, thus facilitating long-term potentiation in the hippocampus (see Table 3).

To support this idea, CGP36742, a $GABA_B$ antagonist, has been shown to improve learning performance in aged rats as well as the performance of rhesus monkeys in conditioned spatial color task. (Mondadori, C. et al., 1993). The significance of the $GABA_BR1/R2$ receptor in cognitive functioning might be indicated by the presence of $GABA_BR2$ mRNA in the cerebral cortex and its codistribution in the ventral forebrain with cortically projecting cholinergic neurons as well as its localization in the pyramidal cells in all regions of Ammon's horn and dentate gyrus in the hippocampus.

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